A REVIEW OF ALTERNATIVES TO CONVENTIONAL MICROSCOPY IN THE LABORATORY DIAGNOSIS OF MALARIA

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DECLARATION

I, Dr G Lokeshwari solemnly declare that the dissertation entitled **"A REVIEW OF ALTERNATIVES TO CONVENTIONAL MICROSCOPY IN THE LABORATORY DIAGNOSIS OF MALARIA"** was done by me at Coimbatore Medical College Hospital, during the period of September 2011 to September 2012 under the guidance and supervision of **Dr V Sadhiqua, M.D.,DGO.,** Associate Professor of Microbiology, Coimbatore Medical College, Coimbatore.

This dissertation is submitted to The Tamilnadu Dr.M.G.R.Medical University towards the partial fulfillment of the requirement for the award of M.D. Degree (Branch - IV) in Microbiology.

I have not submitted this dissertation on any previous occasion to any University for the award of any degree.

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INTRODUCTION Malaria is the disease of antiquity, having been recognized since Vedic times in India 1&2. It is distributed worldwide with a great socioeconomic impact on the countries with intense transmission particularly in tropical regions 3. Now malaria is a reemerging disease and is of major public health concern 4. Patients affected with malaria present with varied clinical picture from simple fever and malaise to life threatening symptoms like cerebral malaria. Thus malaria should be considered in the differential diagnosis of any febrile illness especially in those with a history of travel to malaria endemic places 5. Diagnosing the disease only with clinical features lead to...

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INTRODUCTION

Malaria is the disease of antiquity, having been recognized since Vedic times in India [1&2]. It is distributed worldwide with a great socioeconomic impact on the countries with intense transmission particularly in tropical regions [3]. Now malaria is a reemerging disease and is of major public health concern [4].

Patients affected with malaria present with varied clinical picture from simple fever and malaise to life threatening symptoms like cerebral malaria. Thus malaria should be considered in the differential diagnosis of any febrile illness especially in those with a history of travel to malaria endemic places [5].

Diagnosing the disease only with clinical features lead to over-diagnosis especially in endemic areas. Thus parasite based diagnosis is recommended for all patients by WHO [6]. It urges malaria endemic countries to diagnose the disease by laboratory testing. It also emphasizes on early diagnosis, appropriate anti-malarial treatment and effective surveillance.

A sensitive diagnostic tool is essential to ensure appropriate treatment for the patients. Thus, there is a need to review the currently available diagnostic methods in terms of feasibility in hospitals, rapidity

in diagnosis, cost of the test and its usefulness to patient, laboratory and physician caring the patient.

Common diagnostic procedure followed at Coimbatore Medical College Hospital, Coimbatore is thick and thin smear preparation and staining with Leishman's stain. Though it allows detection, quantification and identification of Plasmodium species, has limited sensitivity when parasitemia is low and in detecting mixed infection.

Thus emphasis has been placed on alternative methods in the diagnosis of malaria. Some of them are Quantitative Buffy Coat, fluorescent microscopy, confocal microscopy, immunochromatography, ELISA, Polymerase Chain Reaction, automated hematology analyzers and microarray.

In this study, Rapid Diagnostic Tests, ELISA for antigen detection and PCR are compared with the Gold standard test 'Microscopy', so that these tests can be used as an alternative in the diagnosis of malaria.

AIMS AND OBJECTIVES

Overall Aim: To find alternatives to Conventional Microscopy in the Laboratory diagnosis of Malaria.

Specific Objectives:

1. To identify malarial parasite in patients with low parasite densities.
2. To compare the sensitivity and specificity of Rapid Diagnostic Test (RDT) with conventional microscopy.
3. To evaluate the diagnostic performance of panLDH Antigen detection ELISA.
4. To evaluate the role of PCR in the diagnosis of malaria.

REVIEW OF LITERATURE

History:

- Malaria has had an enormous impact on human health for millennia. **Hippocrates** described the clinical presentation and complications of malaria in the fifth century BC. Nearly half a millennium later, **Celsus** described the distinctions between the clinical manifestations of disease caused by Plasmodium *falciparum*, Plasmodium *vivax* and Plasmodium *malariae*.
- In **1630s**, it was discovered that the bark of the cinchona tree from Peru could treat the disease. It was imported to Europe and was widely used in treatment. Morton and Sydenham in England and Torti in Italy distinguished between fevers that responded to the bark and those that did not. In England, the responsive fevers were called “agues”. In the **eighteenth century**, they received the Italian name, Malaria (mal aria – bad air), because it was believed that the disease was transmitted through the foul air of swampy areas.
- In **1820**, Pelletier and Caventou determined the structure of quinine and the era of modern chemotherapy was initiated.

- In **1880 Laveran,** a French military physician working in Algeria, made the first description of malaria parasites in the blood of patient and was **awarded noble prize in 1907**.
- In **1883 Marchiafava,** used Methylene blue for staining malarial parasites
- In **1887 Ronald Ross,** a British military physician working in India discovered the transmission of malaria by Anopheles mosquitoes.
- In **1891** Romanowsky introduced polychrome methylene blue for staining malaria parasites.
- In **1898 Ronald Ross,** demonstrated oocyst of Plasmodia on the stomach wall of an anopheline mosquito and later demonstrated lifecycle of malarial parasite in mosquito. He was **awarded noble prize in 1902**.
- In **1898-1899,** Italian scientists **Bignami, Bastianelli and Grassi,** elucidated the life cycle in humans. This led to initiation of control efforts.
- In 1**976, Trager and Jensen** successfully cultivated and maintained Plasmodium *falciparum* in vitro, in human red blood cells [7].

Currently the impact of malaria in many parts of the world is similar to or greater than it was 50 years ago as the campaign of 1950s and 1960s to eradicate malaria came to a halt [8].

On June 13, 2007, the U.S. Food and Drug Administration (FDA) approved the first Rapid Diagnostic Test (RDT) for use in the United States [9].

Serological and molecular diagnostic procedures for detection of the malarial enzymes and nucleic acid respectively have come into existence.

Epidemiology:

Malaria is an overwhelming problem in developing countries especially in the Tropical region [10]. As per the World Malaria Report 2011, malaria is currently endemic over 106 countries. Globally, around 216 million cases of malaria were reported in 2010, leading to approximately 655,000 deaths. Out of this, 81% percent of cases and 91% of deaths occurred in the African region. Mortality was more in children under 5 years of age accounting for about 86% of the total [11].

In India, the annual incidence of malaria is 1.5 million cases [12]. Of this, about 88% of malaria cases and 97% of deaths are reported from North-eastern states. However other states are also vulnerable with local and focal outbreaks of malaria [13].

In TamilNadu, total no. of malaria cases estimated during the year 2008 to 2009 was 3678 [14].

Geographical distribution:

The global distribution and prevalence of malaria have not changed appreciably for the past few decades. It is found in tropical regions of sub-Saharan Africa, many areas of Indian subcontinent, Southeast Asia, Central and South America [8].

Malaria continues to be a major public health threat in India particularly due to Plasmodium *falciparum*. Orissa, Chattisgarh, West Bengal, Jharkhand and Karnataka are the most affected states in India [12].

Out of the total malaria cases in Tamilnadu, 74% are reported in Chennai City and another 8.4% in Tuticorin, Erode, Vellore, Dindigul, Salem, Tiruchengode, Tiruvallur and Tiruvottriyur [15].

There is difference in the distribution of the vectors of malaria and also prevalence of Plasmodium species in different parts of the world.

Vectors:

Anopheles culicifacies is the main vector of rural and peri-urban areas and is widespread in India. It is found in natural and man-made breeding sites. Anopheles stephensi is responsible for malaria in urban and industrial areas (Fig a). Anopheles fluviatilis is the main vector in

hilly areas and forests. Anopheles minimus is the vector in foothills. Anopheles dirus is the vector in the forest of north-east. Anopheles epiroticus in Andaman and Nicobar islands [13]. Anopheles stephensi is the commonest species in Coimbatore.

Plasmodium species:

- Plasmodium *falciparum* is mostly confined to tropical climates and is the major cause of malaria in tropical regions of Africa, north-eastern regions of India and Sri Lanka.
- Plasmodium *vivax* occurs in temperate zones as well as in subtropical areas. It is the wide spread species found in India.
- Plasmodium *ovale* is prevalent in West Africa.
- Plasmodium *malariae* has a limited distribution in India.
- Plasmodium *knowlesi*, the most recently described "zoonotic" species, is restricted to South-East Asia [3,7].

There is increase in malarial cases in non-endemic areas. This is due to immigration, tourism and professional travelling of people from malaria endemic areas [10,16,17]. In the year 2000, a total of 145 cases were imported to Tamil Nadu from Kerala, 121 from Andhra Pradesh and 99 cases from Karnataka [15].

Malaria - an overview of the parasite and the disease: Malaria is caused by Plasmodium, which is transmitted through the bites of infected female anopheles mosquitoes. Five species have shown to infect humans: Plasmodium *falciparum*, Plasmodium *vivax*, Plasmodium *ovale*, Plasmodium *malariae* and Plasmodium *knowlesi* [3&7].

Plasmodium undergoes sexual cycle in the mosquito and an asexual cycle in man. During the bite of an infected mosquito, Plasmodium sporozoites are injected into the blood which moves to liver cells where they develop into liver schizonts. After the rupture of schizonts, thousands of merozoites are released into the bloodstream. Each merozoite invades a red blood cell (RBC). In the RBC, they develop into trophozoites, mature into schizonts. This inturn divide into new merozoites which invade new RBCs. After one or two weeks, gametocytes are produced. These continue the sexual cycle when taken up by a mosquito during the next blood meal. Liver schizonts of Plasmodium *vivax* and Plasmodium *ovale* may persist for months in the liver before releasing merozoites into the blood. The persistent ("dormant") forms are called hypnozoites which cause delayed symptoms resulting in relapse [3&7].

Children, pregnant women, immunosuppressed individuals and non-exposed travellers are particularly vulnerable to malaria [10].

Malaria is also transmitted through blood transfusion and transplacental transmission.

Malaria is characterized by fever with or without periodicity, chills, excessive sweating, malaise, headache, myalgia, arthralgia, anorexia, nausea and vomiting. The symptoms of malaria can be non-specific and mimic illness caused by many viral and bacterial pathogens [8]. If not treated, malaria can become life-threatening. This is especially the case for Plasmodium *falciparum* which unlike Plasmodium *vivax*, Plasmodium *ovale* and Plasmodium *malariae* can infect RBCs of all ages and cause elevated parasite densities. In addition, infected RBCs of Plasmodium *falciparum* stick to the vascular endothelium of the blood vessels in internal organs (sequestration as in the figure b), leading to obstruction in blood flow, resulting in lethal complications such as cerebral malaria, pulmonary oedema and impairment of liver and renal functions [8&18]. Prompt diagnosis is essential for the treatment and outcome of Plasmodium *falciparum*.

Plasmodium *vivax* accounts for almost half of the malaria infections worldwide and is no longer considered as a mild infection. Complicated infections with Plasmodium *vivax* have been demonstrated both in endemic countries and among travellers of non-endemic countries on their return from endemic countries [19].

Fig a: Female Anopheles *stephensi* taking a blood meal

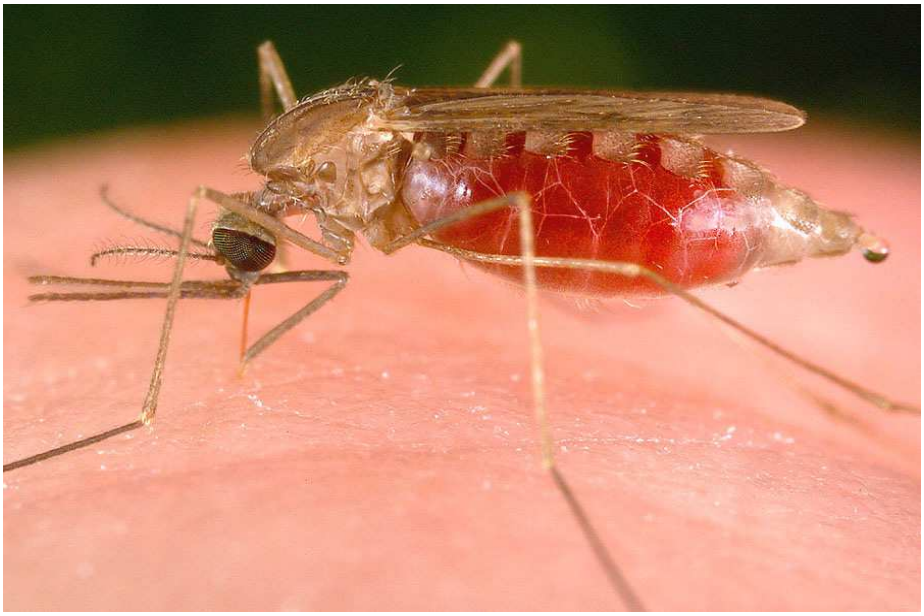
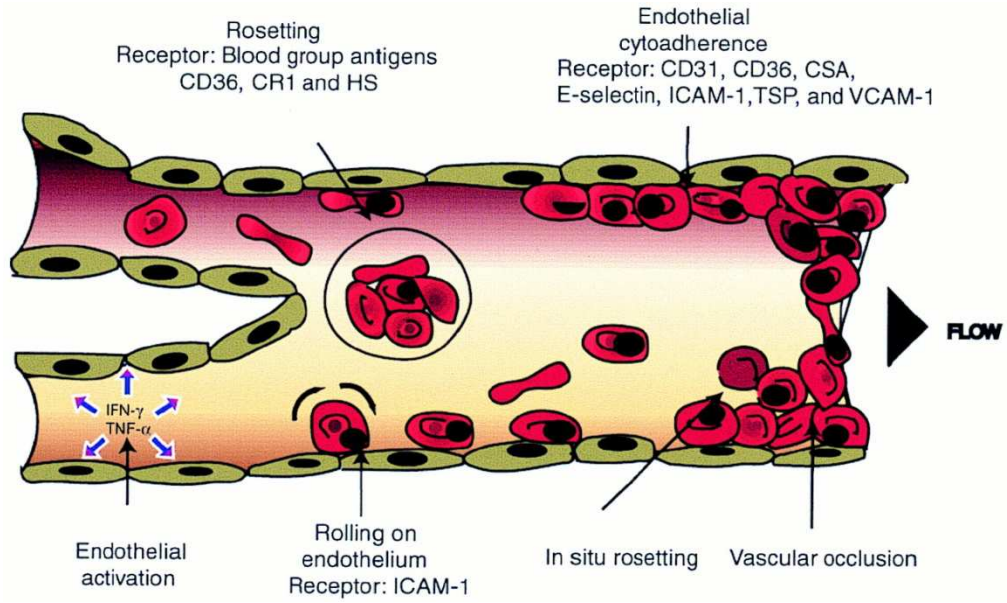


Fig b: Sequestration of RBCs in the blood vessels of internal organs



CD – Cluster of Differentiation, CS - Chondroitin Sulphate A, CR - Complement Receptor, HS - Heparan sulphate, E-selectin – Endothelium selectin, ICAM– Intracellular Adhesion Molecule, TSP - Thrombospondin, VCAM – Vascular Cell Adhesion Molecule.

Emerging threats:

- Plasmodium *falciparum* resistance to artemisinins was first confirmed on the Cambodia-Thailand border in 2009. It has now been identified in Myanmar and Vietnam. WHO has recommended all the countries to ban the marketing of oral artemisinin-based monotherapies, which is one of the major factors fostering the emergence and spread of resistance [20].
- The problem of insecticide resistant mosquitoes is increasing. Four classes of insecticides are organo-chlorine compounds like DDT, organo-phosphorus insecticides like malathion, parathion, abate, carbamates like carbaryl, propoxur and synthetic pyrethroids like resmethrin. **According to the World malaria report 2011**, 45 countries around the world have identified resistance to at least one of the four classes of insecticides used for malaria vector control. India and malaria-endemic countries in Sub-Saharan Africa are one among them [20].

With the emergence of insecticide resistance in vectors, the National Malaria Eradication Programme came to a standstill. There was resurgence of malaria in early 1970, following which many programmes like National antimalarial programme, National Vector Borne Disease Control Programme, Introduction of RDT programme, Artemesinin

Combination Therapy (ACT), Introduction of Long Lasting Insecticide Nets to control malaria came into existence [13].

Diagnosis of malaria:

Malaria is diagnosed by clinical, parasitological, immunological and molecular methods. Malaria can mimic many diseases. The golden rule is to always include malaria as one of the differential diagnosis of a febrile illness without any focus of infection.

In the pre-eradication era (prior to National Malaria Eradication Programme, 1958), spleen rate, average enlarged spleen, were used to measure the endemicity of malaria in the community. These were found nonspecific in tropical countries because splenomegaly is also seen in many cases other than malaria [13].

Intensive research on various diagnostic tools of malaria has resulted in improved diagnostic procedures. The aim of these procedures is to provide simple and rapid non-microscopic diagnostic methods with increased sensitivity for detecting the parasite.

Purpose of an appropriate diagnostic method is to rule out malaria in symptomatic patients, to guide the physician in starting an appropriate treatment and to help in epidemiological studies.

Diagnostic methods:

1. **Microscopy:**
    i. Staining of Thick and thin blood films with Romanowsky's stains followed by examination under light microscope.
    ii. Fluorescent microscopy or a microscope with an interference filter system (Kawamato method) - used to examine quantitative buffy coat and acridine orange stained smears.
    iii. Confocal microscopy – A microscope system which removes out-of focus information optically. It provides better resolution image with high contrast and reduced glare. 3-D reconstruction is also possible with this microscopy [21]. It helps to visualize live intra-erythrocytic malarial parasite, to quantify malarial pigments and to find out the antimalarial drug resistance.
2. **Quantitative Buffy Coat (QBC) :**

Here the blood is centrifuged in a specially designed microcapillary tube fitted with a plastic float. The tube is precoated with fluorescent dye. The float spreads the buffy coat against the edge of the tube. Parasite and the leucocytes take up the dye, which is fluorescent when examined under ultraviolet light in a fluorescent microscopy. QBC is slightly more sensitive than thick films but has some limitations: species identification

and quantification are difficult. It requires microcapillary tubes and fluorescent microscopy, which are expensive. QBC cannot be stored for later reference [3&22].

3. Immunological methods:

Immunological methods detect parasite antigens or the host antibodies directed against the parasite. The detection of antigens is an acceptable alternative to parasite detection. The detection of antibodies is of limited use for individual diagnosis as they cannot distinguish between current and past infection.

A good antigen detection test should detect only the current infection. It should not produce any false negatives for it to be highly sensitive. It should produce fewer false positives for it to be highly specific [3].

ELISA, Radioimmunoassay, immunochromatography are some of the methods to detect malarial antigens [3].

4. Molecular methods:

The application of molecular methods to diagnose malaria has several advantages over traditional methods. It serves as a research tool to monitor malaria control programs and to perform quality control checks on microscopic diagnosis. It helps to determine the distribution of important genes such as genes associated with drug resistance.

Sensitivity of nucleic acid probes has increased exponentially with the availability of amplification methods such as Polymerase chain reaction.

5. Newer advances in the diagnosis of malaria:

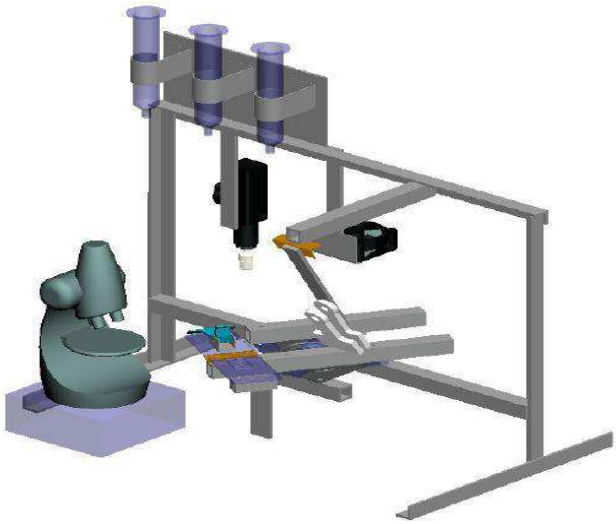
i. Microarrays: Microarrays are collection of nucleic acid sequences immobilized onto a solid support (slide). The basis of Microarray is classical Northern blotting – base pairing and hybridization between nucleic acids. Each unique sequence form a tiny feature, called a 'spot' or 'target'. Target in the sample is labelled with fluorescent dyes. Following hybridization, the slide is scanned with the help of LASER scanner to generate images. The image is then analyzed with the help of microarray image analysis software.

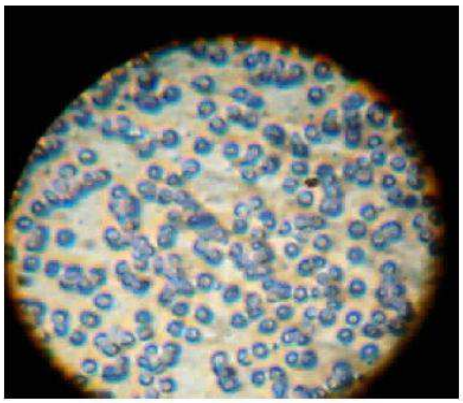
Microarray differs from northern blotting in detecting thousands of genes simultaneously. It helps in detecting different stages of intra-erythrocytic development and drug resistance [23].

ii. Automated parasite detection system(APDS):

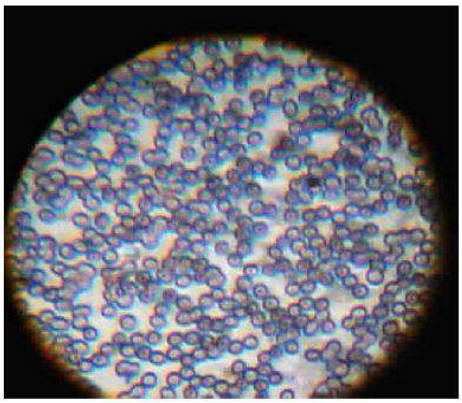
Automated Giemsa staining apparatus creates a thin smear, fixes the cells with methanol, stains with Giemsa stain, rinses and dries the slide and inspects the slide under microscope. The data is acquired in the computer. Computer algorithm determines the presence or absence of malaria [23] (Fig c).

Fig c: Automated Parasite Detection System





Uninfected Red Blood Cells



Infected Red Blood Cells

Advantages of APDS are low false-positive & false-negative rates. It can diagnose malaria within 60 minutes. It is a portable device, thus easy to transport. It will serve as a low-cost, easily accessible malaria testing device in remote areas, thus decreasing mortality rates.

iii. **Malaria detection by haematology analysers**, as a method of malaria parasite detection came into reality only after the 1990s. But false positivity and high cost limit their use [23].

iv. **Automated Microscopy Diagnosis** (Computer Vision For Microscopy):

- Underlying principle: Ability to differentiate between non-parasitic stained components/bodies (RBC's, WBC's, platelets, artefacts) and the malaria parasite using visual information (Visual Basic Computer language).
- It requires combination of microscopy, digital photography, and computer software.
- Its most common application is in the diagnosis of malaria [23].

v. **Automated nucleic acid extraction:**

Fully automated DNA extraction techniques are now available which helps to process a large number of samples [23]. This is of great help in RT-PCR.

In this study, the diagnostic tools reviewed and performed were peripheral smear and microscopy, rapid diagnostic tests, ELISA and Real time PCR.

Blood smears:

Conventional Microscopic examination of peripheral smear is accepted as the universal "gold standard" for the diagnosis of malaria [7&22]. Both thick and thin smears are prepared from the capillary blood or anticoagulated venous blood. They are stained with one of the Romanowsky's stains such as Leishman's stain, Jaswantsingh bhattacharji stain, Giemsa, Wright's, or Field's stain [18]. Different stages of the parasite like ring forms, schizonts and gametocytes can be visualized [18].

Microscopic examination of the blood smear can detect the parasites, quantitate parasitemia and demonstrate malarial pigments.

Thick smear:

During the preparation of thick smear, concentrated red blood cells are lysed with distilled water. Smear is dried thoroughly and stained without fixing [7].

Its thickness should be such that it contains 10 layers of RBCs. Approximately 10 to 12 WBCs should be visible per oil immersion field[24]. Threshold of detection of malarial parasite in thick film is 5 to 20

parasites/µl of blood. At least 100 to 200 fields, each containing 20 WBCs should be examined before a thick smear is reported as negative for malaria [7].

Advantages of thick smear are - it allows examination of greater volume of blood. As the parasites are concentrated, it detects low levels of parasitemia especially during recrudescence or relapse [22&25]. It is 30 times more sensitive than thin smear [7].

Disadvantages are – it does not help in speciation as the parasites in the lysed cells are distorted. So laboratories with limited experience may prefer thin smear [22&25].

Thin smear:

The thin smear consists of a single layer of RBCs. Thin smears are air-dried, rapidly fixed in alcohol and stained (alcohol fixation not needed for Leishman's stain). The RBCs in the tail end of the smear are examined for the parasite. Threshold for detection of parasites with thin film is 100 parasites/µl of blood.

Thin smear helps to speciate, identify the stage of the parasite and to assess the response to treatment especially in areas where drug resistant falciparum is suspected.

The disadvantage with thin smear is that it is less sensitive than a thick film when there is low parasitemia.

Advantages of microscopy are it is Simple and cheap. It can identify the presence of parasites, the infecting species, stage of the parasite and assess the parasite density.

Limitations of microscopy are: labour intensive, time consuming, require expertise and trained healthcare workers. It is not suitable for examination of large number of blood smears. The sensitivity of detecting the parasite decreases with low parasitemia particularly in falciparum malaria where sequestration of the parasites occurs in the capillaries of the internal organs and in mixed malarial infection [26].

An expert microscopist detects the parasite even when its level in the blood is around 5 parasites/μl of blood. But, an average microscopist detects the parasite only when the level of the parasite is around 50-100/μl of blood. This has probably resulted in underestimation of malaria infection rates, especially in cases with low parasitemia and asymptomatic malaria [27].

Intradermal smears:

Smears prepared from intradermal blood have been shown to contain more mature forms of Plasmodium *falciparum* than peripheral blood [1].

Rapid diagnostic tests:

Immunochromatography tests are based on the capture of the parasitic antigens from the peripheral blood using either monoclonal or

polyclonal antibodies. Currently, immunochromatography tests target the Histidine-Rich Protein 2 (HRP2) of Plasmodium *falciparum,* a pan-malarial Plasmodium aldolase, and the parasite specific or pan-malarial Lactate Dehydrogenase (pLDH). In India, NVBDCP emphasize on use of RDT in the diagnosis of malaria especially Plasmodium falciparum malaria to improve diagnostic efficiency in Peripheral Health Centres [28].

Histidine-Rich Protein (HRP):

Plasmodium *falciparum* infected RBCs synthesize three Histidine-rich proteins namely HRP-1, HRP-2 and HRP-3. HRP-1 was identified in all knob-positive Plasmodium *falciparum* but only small amounts were present in culture-adapted strains. HRP-2 was identified in all Plasmodium *falciparum* parasites regardless of the knob phenotype and recovered from culture supernatants as a secreted water soluble protein. HRP-3 was present at the lowest concentration when compared to HRP-1 and HRP-2. Neither HRP-1 nor HRP-2 was found in non-falciparum malaria [25].

Histidine-rich protein 2 of Plasmodium *falciparum* (PfHRP2) is a water soluble protein produced by asexual stages and young gametocytes of Plasmodium *falciparum*, expressed on the internal surface of the red cell membrane and also secreted into the blood [3]. It remains in the blood for at least 28 days after the initiation of antimalarial therapy [1&25].

Several RDTs targeting PfHRP2 have been developed which are highly sensitive but false negatives have been reported in patients whose parasites have a rare antigenically variant form of PfHRP2 [1].

Plasmodium specific Lactate Dehydrogenase:

Plasmodium is a homolactate fermenter. It depends extensively upon anaerobic glucose metabolism for ATP production. Parasite LDH is a 316 amino acid soluble glycolytic enzyme produced by the asexual and sexual stages of the live parasites and released from the infected erythrocytes. It converts pyruvate to lactate while regenerating NAD+ for continued use in glycolysis. pLDH has notable structural and kinetic properties making it different from mammalian and bacterial LDH enzymes [29]. Parasites should be viable for pLDH to be detected. pLDH with different isomers exist for each species [22&30].

With pLDH as the target, quantitative immunocapture assay, qualitative ELISA, qualitative immunochromatographic card tests using monoclonal/polyclonal antibodies, immunodot assays, and dipstick assays using polyclonal antibodies have been developed.

pLDH is superior to HRP-2 for its shorter shelf life in blood. pLDH clears at about the same time as the parasites, following successful treatment, thus useful for monitoring responses to drug therapy and

detecting drug-resistant malaria because the level of pLDH correlates with the number of viable malarial parasites in the blood.

Plasmodium specific aldolase:

Plasmodium aldolase is an enzyme of the parasite glycolytic pathway expressed by the erythrocytic stages of Plasmodium *falciparum* as well as the non-falciparum malarial parasites in the blood. Monoclonal antibodies against Plasmodium aldolase are pan-specific in their reaction and have been used in the combined 'P.f/P.v' immunochromatographic test that targets the pan malarial antigen (PMA) along with PfHRP2.

The RDTs have been developed in different test formats like the dipstick, strip, card, cassette and the latter has provided a more satisfactory device for safety and it is user friendly. The test procedure varies between the test kits. In general, the blood specimen: finger-prick blood specimen or anticoagulated blood or plasma is added to the sample well and lysing/washing buffer is added. Labelled antibody is pre-deposited in the cassette during manufacture. If the target antigen is present in the blood, a labelled antigen-antibody complex is formed and it migrates up the test strip to be captured by the pre-deposited capture antibodies specific against the antigens and against the labelled antibody

(as a procedural control). Coloured lines are formed by the immobilized antigen-antibody complexes.

Immuno-enzymatic tests are of immense help in patients with clinical suspicion of severe falciparum malaria and in detecting mixed Plasmodium *falciparum* and Plasmodium *vivax* infections where parasites (Plasmodium *falciparum*) are not detected microscopically because the parasites are sequestered in the blood vessels of internal organs [1&26].

RDTs are rapid, simple to perform and easy to interpret [31]. One test can be completed in 10-15 minutes and many can be done simultaneously. They are highly sensitive and specific. It does not require expertise, equipment, or laboratory facilities. Unskilled staff with only a little training can perform the test and hence most suitable for field conditions [32].

Thus it is possible to detect malarial parasites even in peripheral settings where there is a lack of basic equipments and infrastructure. These rapid tests can be performed and interpreted by primary health care workers. This in turn helps in early and appropriate management of malaria in Low level health care facilities [33&34].

There exist certain limitations for RDT. The sensitivity of RDT to detect malarial parasite is good at parasite densities >500/µl of blood but, sensitivity decreases as the parasitic density decreases below this [35].

It cannot quantify the parasite density or differentiate sexual and asexual stages of the parasite. It is of relatively high cost. False positivity due to persistence of PfHRP2 and cross reactivity with rheumatoid factor can occur.

Reasons for persistent PfHRP2:

1. Persistent viable asexual stage parasitemia below the detection limit of microscopy
2. Delayed clearance of circulating antigen and
3. Persistent sexual stage forms following treatment [36,37].

ELISA for antigen detection:

The malarial antigens pLDH can also be detected by Enzyme linked immunosorbent assay.

Principle:

The pLDH antigen in the sample is detected by direct sandwich ELISA. Here the antigen in the sample binds to the monoclonal antibody coated in the microtitre well. When a second monoclonal antibody linked to biotin is added, it binds to immobilized antigen. The addition of streptavidin-HRP conjugate results in binding of the conjugate to biotin. When substrate is added, the HRP enzyme cleaves the substrate and results in formation of coloured product. The rate of colour formation is directly proportional to the amount of antigen in the sample.

Advantages of ELISA are simple to perform, large number of samples can be tested in a single test run within a short time frame and it is quantifiable [38].

A disadvantage in using pan-specific LDH antibodies coated microwell for detecting malaria is - speciation cannot be done.

Polymerase Chain Reaction:

Molecular methods based on DNA amplification have been applied to malaria diagnosis since the late 1980s [39]. PCR showed high sensitivity by detecting ≤ 5 parasites / µl [25]. PCR is also highly specific and detects drug resistance in Plasmodium *falciparum* [25]. Conventional PCR is being widely used. With conventional PCR, carry over contamination during the manipulation of postamplication products can occur [40].

Real-time PCR methods are a recent development of the PCR methodologies. This new technique is based on fluorescence. Real-time PCR assays have closed amplification vessels for thermo-cycling, amplicon monitoring and detection. Amplicon (amplified target) is monitored as it gets generated. Monitoring of the amplified target is made possible by labelling of oligonucleotide probes with molecules capable of fluorescing. This decreases the need for excessive handling of samples, particularly the post-PCR handling of samples to detect the amplicons. These features significantly reduce the sample contamination. The

internal control provided in the kit helps to monitor the quality of DNA extraction and the PCR run, thus helps the user to monitor for PCR inhibition [41].

RT-PCR has the ability to detect multiple targets. This is made possible by using different probes labelled with specific fluorescent dye each having their own unique emission spectra [42].Thus multiple species can be identified in one run. This reduces carry over contamination and less time consuming.

Genes targeted for species level differentiation of Plasmodium are the circumsporozoite genes and the 18SrRNA genes.

PCR can also detect infection due to *Plasmodium knowlesi*, the newly discovered fifth species of Plasmodium responsible for human disease [27].

PCR can identify, speciate, quantify the malarial parasites. PCR can detect malaria even in asymptomatic subjects with submicroscopic parasite densities, who are the source of transmission and hindrance to elimination of malaria [43,44]. It plays an important role in detecting drug resistant parasites [39].

Advantages of RT-PCR:

1. Reduced risk of contamination when compared to conventional methods.

2. Able to arrive at the result within 3 hours (including DNA extraction) [39].
3. Automated PCR can process large number of samples.

Limitations are it involves complex methodologies, costlier, needs specially trained technicians [27]. It is not suitable for diagnosis of malaria in rural areas [45].

Thus understanding the characteristics of malarial parasites, use of appropriate diagnostic procedures and a complete understanding of the limitations of each procedure is very important to choose an alternative diagnostic tool in the laboratory diagnosis of malaria.

Early diagnosis helps in initiation of timely and appropriate treatment. This inturn prevents progression of uncomplicated malaria to severe disease, prevent death, interrupt transmission and minimize the spread of drug resistant parasite.

Treatment:

Treatment of malaria is primarily based on specific antimalarial chemotherapy and supportive therapy.

Antimalarial drug should aim at:

- Effective clinical cure by destroying all asexual forms of the parasite in the blood.

- Preventing relapse by destroying hypnozoites and extra-erythrocytic forms.
- Preventing transmission of infection to mosquitoes by destroying gametocytes.

Commonly followed regimen in India according to National Vector Borne Disease Control Programme is Chloroquine and Primaquine for Plasmodium *vivax*, Artemesinin Combination Therapy for Plasmodium *falciparum*.

Prevention and control:

The preventive measures to control malaria mainly depend on treatment of infected individuals and reducing the transmission of malaria.

The control measures include:

1. Control of mosquito population
2. Prevention of mosquito bites
3. Chemoprophylaxis
4. Vaccines

Control of mosquito population:

Adult mosquitoes are controlled by spraying residual insecticides such as Abate. The breeding of mosquito larvae is prevented by eliminating breeding places like swamps, flooding and flushing of

breeding places, spraying the breeding sites with oils and Paris green[13]. Larvicidal fishes like Gambusia feed on mosquito larvae. Bacillus thuringiensis var israelensis H14 produces toxins which kills the larvae. Thus they are used as biological control.

Newer techniques like sterile insect technique which makes the male mosquitoes sterile and transgenic techniques which result in embryonic lethality are under field trial to control vector population [46].

Prevention of mosquito bites:

Mosquito nets, protective clothing, mosquito repellants and Long Lasting Insecticide Lined Nets (LLILN) help prevent mosquito bite [7&13].

Chemoprophylaxis:

Chemoprophylaxis is recommended for travellers, migrant labourers and military personnel visiting endemic areas. The drugs commonly used are Chloroquine, Primaquine, proguanil, amodiaquine, mefloquine and fancidar in weekly doses for long term prophylaxis and doxycycline once daily doses for short term prophylaxis.

The following is the chemoprophylaxis recommended by NVBDCP, Delhi.

Short-term chemoprophylaxis (less than 6 weeks):

Doxycycline: 100 mg once a day for adults and 1.5 mg/kg body weight for children more than 8 years old. The drug should be started 2 days

before travel and continued for 4 weeks after leaving the malaria endemic area.

Long-term chemoprophylaxis (more than 6 weeks):

Mefloquine: 5 mg/kg body weight (up to 250 mg) weekly and should be administered two weeks before, during and four weeks after leaving the malaria endemic area.

Note: Doxycycline is contraindicated in pregnant and lactating women and children less than 8 years. Mefloquine is contraindicated in cases with history of convulsions, neuropsychiatric problems and cardiac conditions [12].

Vaccines:

Most of the malarial vaccines developed are against Plasmodium falciparum, as it causes majority of deaths. Based on the stage against which the vaccines are directed, there are 4 types of malarial vaccines. They are Pre-erythrocytic vaccine candidates, Blood-stage vaccine candidates (Asexual stage), Transmission-blocking vaccine candidates (Sexual stage) and Multi stage vaccines. All the vaccines are under trial.

The vaccine currently in phase III trial is RTS,S. It is a pre-erythrocytic vaccine against Plasmodium *falciparum*, which inhibits the entry of parasite into the liver cells [47]. It is a hybrid molecule expressed in Saccharomyces cerevisiae.

The vaccine RTS,S consist of :

- Tandem repeat tetrapeptide (R)
- C-terminal T-cell epitope containing (T) regions of CSP
- Hepatitis B surface antigen (S) and
- Unfused S antigen (S).
- ASO1: Liposomal formulation as adjuvant.

It induces both humoral and cell mediated immunity against the parasite [46].

MATERIALS AND METHODS

This study “A review of alternatives to Conventional Microscopy in the Laboratory diagnosis of Malaria’’ is a cross-sectional study conducted in the Department of Microbiology at Coimbatore Medical College Hospital, Coimbatore from September 2011 to September 2012.

This study was undertaken after the acceptance of the proposal by Ethics committee. The study group included patients of all age groups and both sexes who were clinically suspected to have malarial fever (both inpatients and outpatients).

No. of patients studied: 242.

Informed consent was obtained from all the patients enrolled in the study. Blood samples from these 242 patients were collected in a vacutainer with EDTA anticoagulant. Leishman’s staining of the peripheral smear, Rapid diagnostic test, ELISA for parasite specific pan malarial antigen (LDH) detection were done on all the samples. PCR was done for a total of 40 samples (20 microscopy positive and 20 microscopy negative samples).

Collection, Transport and storage of blood samples:

Under strict aseptic precautions, 3ml of peripheral venous blood was collected in a vacutainer with EDTA from patients clinically suspected to have malaria before administration of antimalarial drugs.

The blood samples thus collected were transported to the laboratory immediately.

Thick and thin smears were prepared and stained with Leishman's stain. Immunochromatography test was performed. Remaining blood was aliquot for ELISA and PCR. Blood aliquot for ELISA were stored at 4 - 8 ^{0} C and test performed within 24 hours of sample collection. PCR was performed on 20 randomly selected Peripheral smear positive samples and 20 Peripheral smear negative samples. The selection of peripheral smear negative sample was based on the clinical history of the patient which should be suggestive of malaria and other investigations for identifying the cause of fever should be normal. Plasma was separated from the blood aliquot for PCR and RT-PCR was performed.

Peripheral smear: Thick and thin blood smears were prepared from venous blood on a clean, dry, grease free glass slide.

Staining of thin smear with Leishman's stain:

Procedure:

The air dried thin smear was covered with Leishman's stain and allowed to stand for one minute. The stain was then diluted with twice its volume of distilled water. The diluted stain was allowed to remain on the slide for 10 minutes. The slide was held under an open tap and the stain flushed with gentle flow of water. The reverse side of the slide cleaned

with a wet and squeezed cotton wool. The slide was kept in an upright position to drain and dry. The stained and dried film was examined with oil-immersion objective of light microscope.

Staining of thick smear:

The thick smear dehemoglobinised with distilled water, stained with Leishman's stain as for thin smear and examined under oil-immersion objective of light microscope.

Rapid diagnostic test:

Procedure: All the kit components were brought to room temperature prior to testing. The test device was taken out of the foil pouch and placed on a flat, dry surface. The circular end of a disposable specimen loop (capacity 5µl) provided in the kit was dipped into the blood and carefully placed into the round sample well. Four drops of assay diluent was added to the diluent well. The result was interpreted within 20 to 30 minutes by viewing through the result window.

Interpretation:

Positive result:

Plasmodium falciparum infection: Appearance of color band in the T1 region together with a band in the C region.

Non-falciparum malarial infection: Appearance of color bands in the T2 region together with a band in the C region.

Plasmodium falciparum or a mixed infection of Plasmodium falciparum with other species: Appearance of color bands in T1, T2 region together with a band in the C region.

Negative result:

Appearance of a color band in the C region with no bands in any of the T regions indicates a negative result.

Invalid result:

Failure of appearance of color band in the C region indicates an invalid test.

Enzyme Linked Immunosorbent Assay for the detection of parasite specific pan malarial antigen (pLDH):

Procedure:

All the reagents and specimen were brought to room temperature. Required numbers of microwells were taken out of the pouch. Datasheet was prepared and the location of the controls and specimen recorded. Two positive controls and two negative controls were used per test run.100µl of sample diluent containing diluted antibody reagent was added to each well. 25µl of positive control, negative control, sample(whole blood) were added to the respective wells. Microwell plate was gently shaken to mix the contents; microwells in the plate were sealed and incubated at 37^0C for 30 minutes. Each well was washed with

350µl of diluted wash buffer for six times and blotted dry. 100µl of diluted conjugate was added to each well and incubated at room temperature for 30 minutes. Each well was washed with 350µl of diluted wash buffer for six times and blotted dry. 100µl of substrate was added to each well and incubated at room temperature away from light for 30minutes. 100µl of stop solution was added to each well in the same order as that of substrate to stop the reaction. The absorbance of each well was read at 450nm with 600-700nm as reference within 30 minutes of stopping the reaction.

Cutt-off value calculation:

Cutt-off value = 0.1+Average of Negative control.

Interpretation:

- Samples with absorbance value more than the cutoff value: REACTIVE.
- Samples with absorbance value less than the cutoff value: NON-REACTIVE.

Reactive samples are retested in duplicate. If both or either of the duplicate is reactive, the probability of malaria infection is high.

Polymerase Chain Reaction:

Genomic DNA extraction: A 1.5 ml tube was taken, 20µl of Proteinase K was added followed by addition of 200µl of plasma and

200 µl of binding buffer. The tube was then vortexed well and incubated at 60^0C for 10 minutes. 100 µl of isopropranol was added and mixed well by pipetting. The lysate (Proteinase K + Plasma + Isopropranol) was then transferred to the upper reservoir of the binding column tube (fit in a 2ml tube) without wetting the rim. The tube was closed and then centrifuged at 8,000 rpm for one minute. The binding column tube was then transferred to a new 2 ml tube. 500 µl of washing buffer (1) was added to the binding column tube and centrifuged at 8,000 rpm for one minute. The solution collected in the 2ml tube was poured into a disposable bottle. Washing buffer (2) was then added to the binding column tube and centrifuged at 12,000 rpm for one minute. The binding column tube was then transferred to a new 1.5 conical bottom elution tube. 200µl of elution buffer added to binding column tube allowed to remain in the tube for one minute. The tube was centrifuged at 8,000 rpm for one minute. Approximately 200 µl of eluent was collected in the elution tube.

Preparation for PCR amplification:

Desired numbers of PCR tubes were placed in the cooling block. All the reagents were thawed completely and mixed by brief vortexing.

The Real-time PCR was performed based on Taq-man principle. Malaria supermix which contains dNTPs, primers and probes is provided in a ready to use form. Master mix was prepared as in the flow chart

below. 15 µl of the master mix was added to each PCR tube. 10 µl of the DNA extract was added to appropriate sample tubes. 10 µl of standards added to positive control tube and 10 µl of PCR grade water added to negative control tube. The master mix and the DNA extract or standard thus added are mixed well by pipeting up and down. The PCR tubes closed and transferred to the rotor of the Rotor Gene 6000 instrument. Locking ring placed on the top of the rotor to prevent accidental opening of the tubes during the run. The thermal conditions were set as given in the table below. Data acquiring channel Green (FAM) and yellow (Joe) were set after setting the anealing temperature. Test was run. The data analysis performed using Rotor Gene™ software according to the manufacturer's instructions.

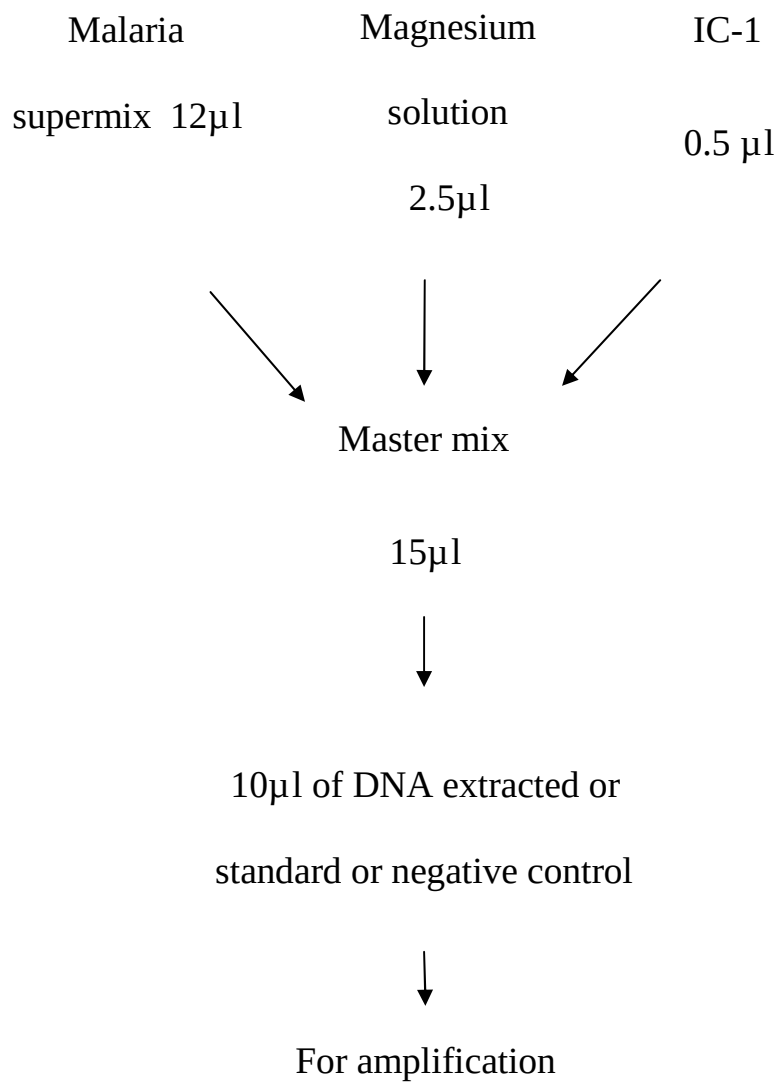
Interpretation:

- If a signal is detected in fluorescence channel cycling A Green, sigmoid shaped curve is generated. If the Ct value is less than or equal to 37, the result of the analysis is positive i.e., the sample contains malaria DNA. Ct value more than 37 is considered as negative.

  Ct value: The cycle number when a sample crosses a threshold value is Ct value.

- If no signal is detected in fluorescence channel cycling A Green, the curve will be just above the threshold level or below the threshold level. It should be associated with amplification in yellow channel (which is due to internal control). The result of the analysis is negative i.e., the sample does not contain malaria DNA.

Preparation of master mix (as per the manufacturer's instructions):



PCR thermal setting

	Time	Temperature
Initial denaturation	10 min	95^0C
3 step cycling		
Denaturation	15 sec	95^0C
Anealing	20 sec	55^0C
Extension	15 sec	72^0C

Repeat cycle 45 times

Figure (i): Peripheral smear – Thick and thin smear

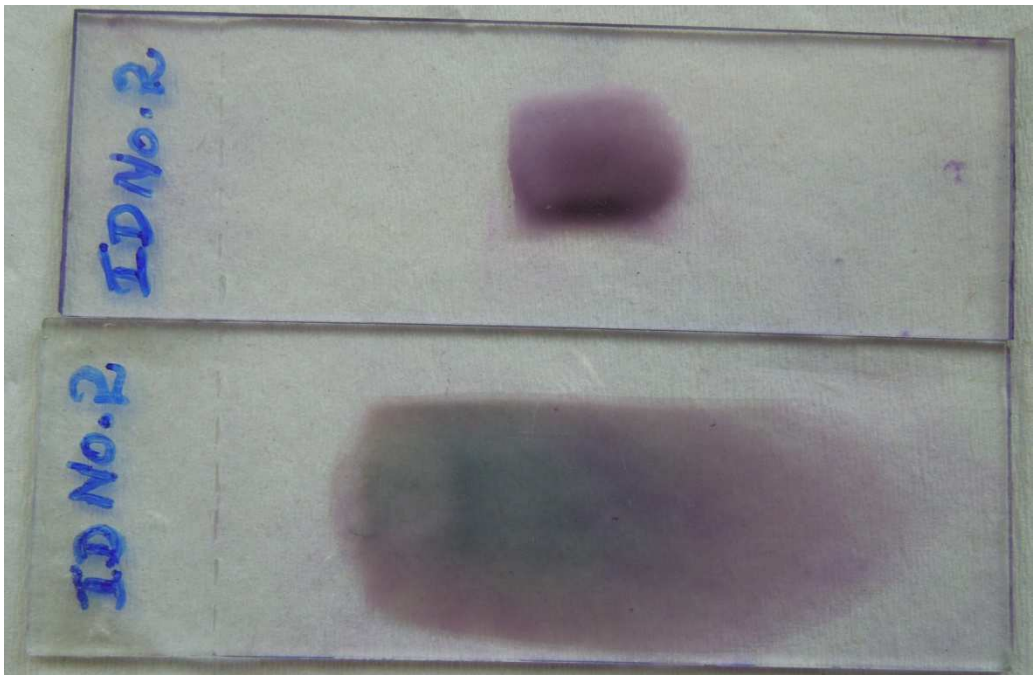


Figure (ii): Peripheral smear of a 1 year old male child with fever for a week showing early ring forms (1,2) of Plasmodium vivax.

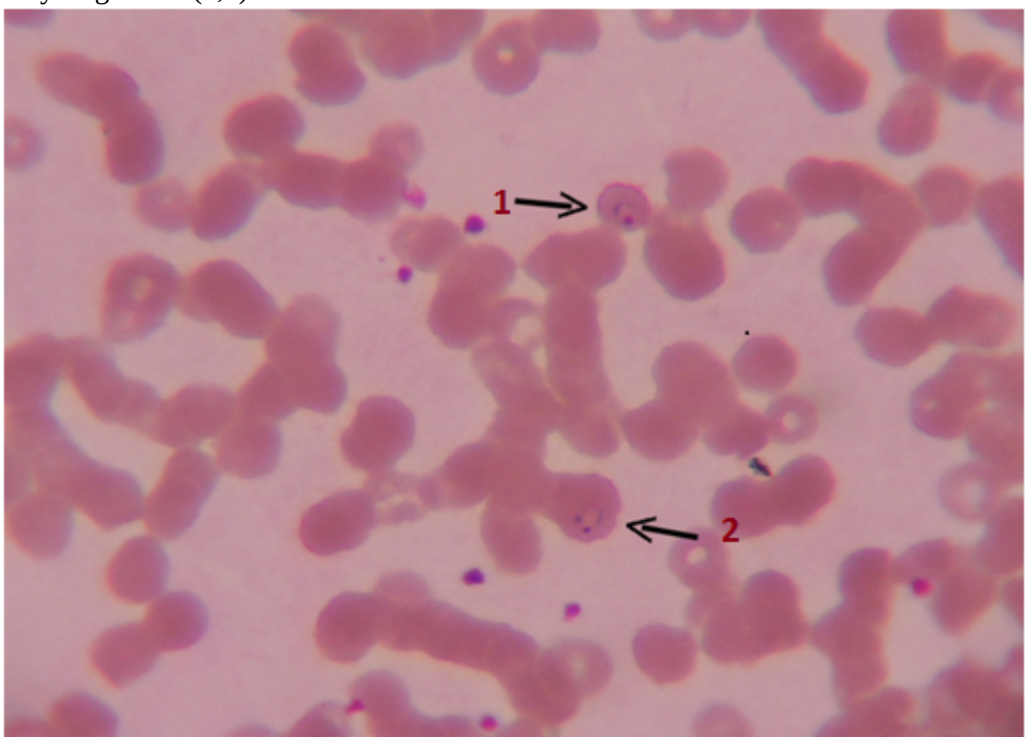


Figure (iii): Peripheral smear of a 45 year old male with fever for 10 days showing Ring forms (1,2,3) of Plasmodium vivax with Schuffner's dots and enlarged RBCs

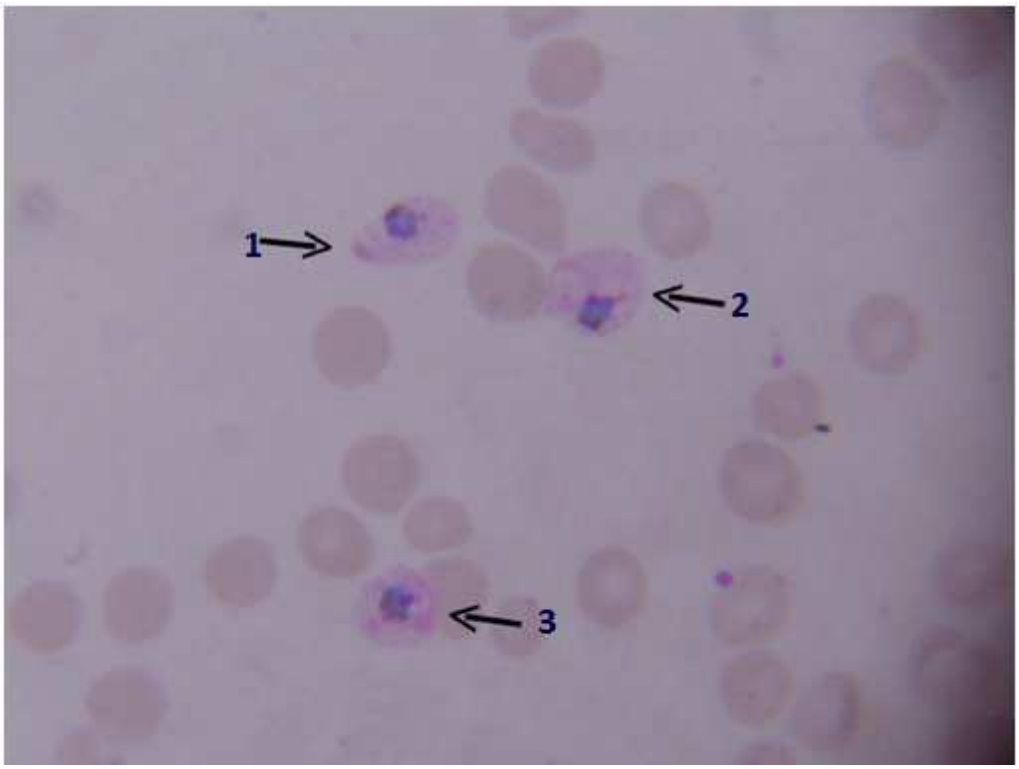


Figure (iv): Peripheral smear of a 51 year male with fever, rigors and headache for 5 days showing amoeboid form(2) and Early ring form (1) of Plasmodium vivax.

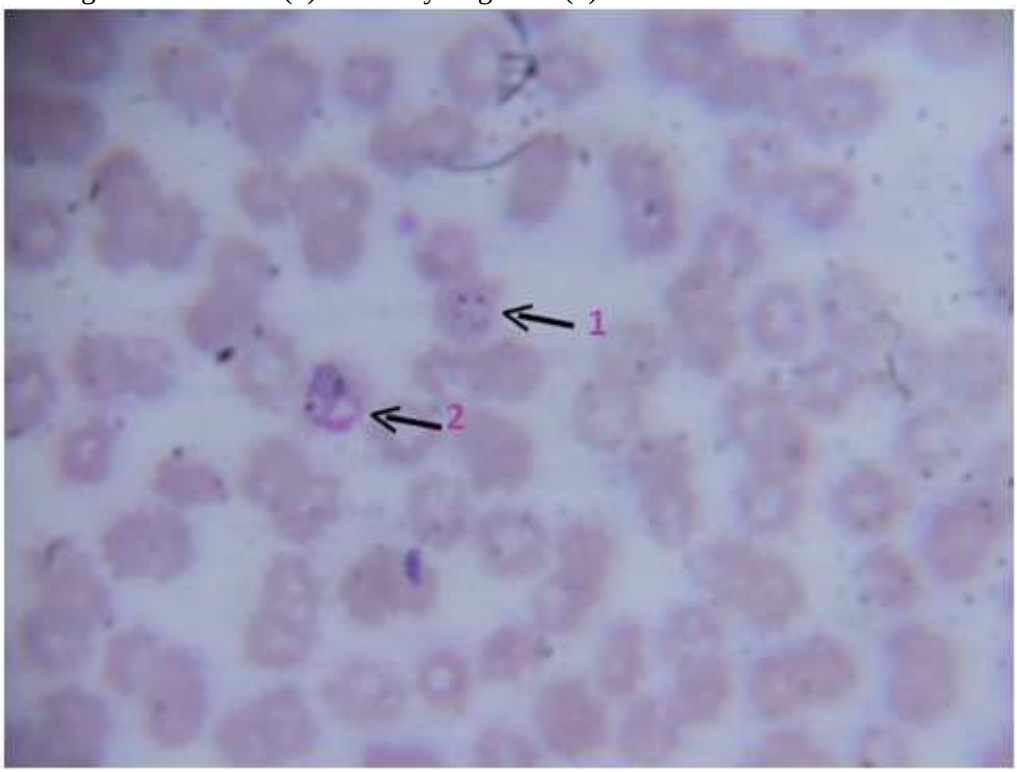


Figure (v): Peripheral smear of the same patient showing amoeboid form of Plasmodium vivax with schufners dots(1).

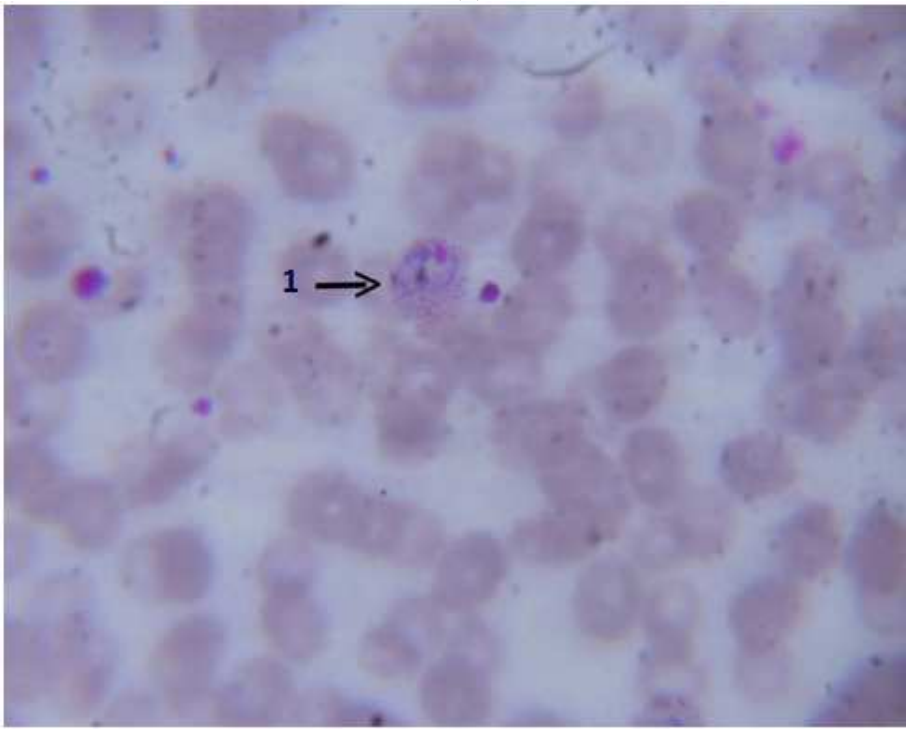


Figure (vi): Peripheral smear of a 54 year male with fever for 10 days showing early schizont(1) and early ring form (2) of Plasmodium vivax.

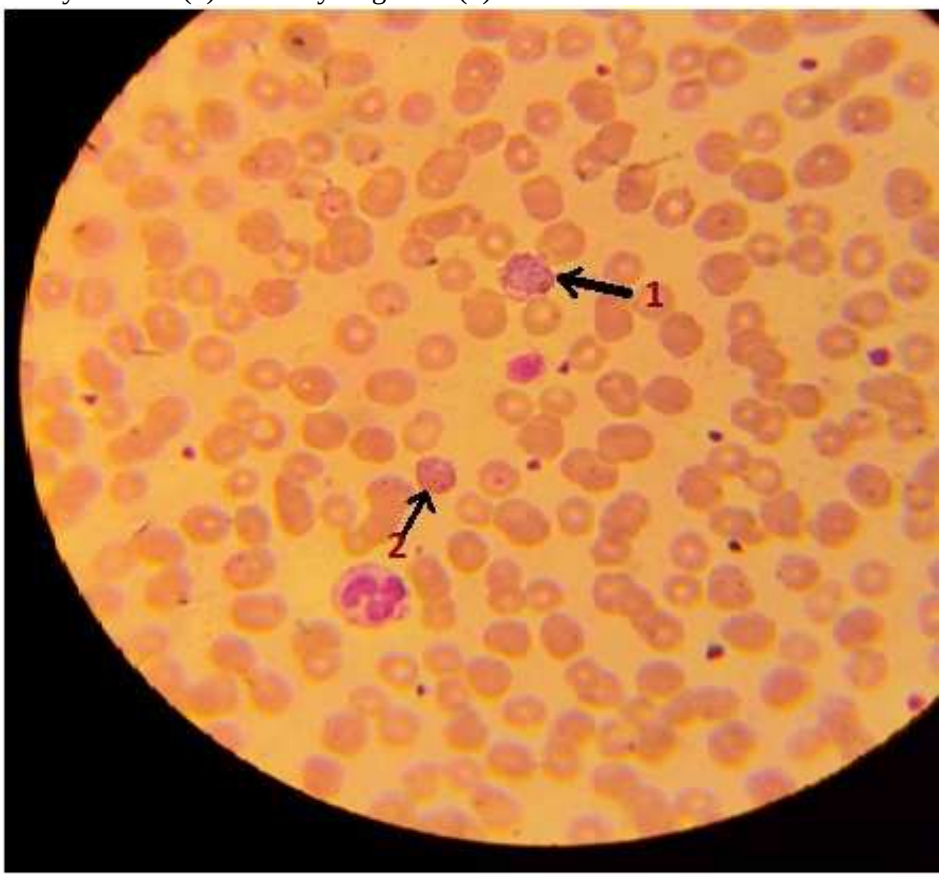


Figure (vii): Peripheral smear of a 3 year old female child with cerebral malaria showing early schizonts(2), Trophozoite(1) and early ring form(3) of Plasmodium vivax with enlarged RBCs.

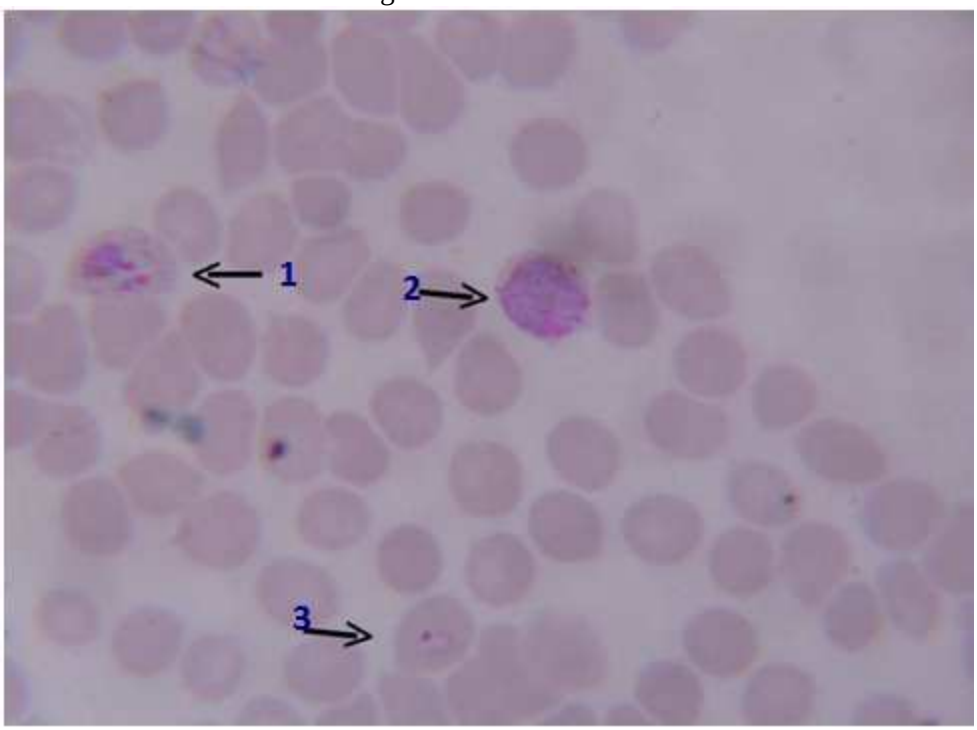


Figure (viii): Peripheral smear of the same patient showing mature schizont (1), Trophozoite(2) and early ring form(3) of Plasmodium vivax and enlarge RBCs.

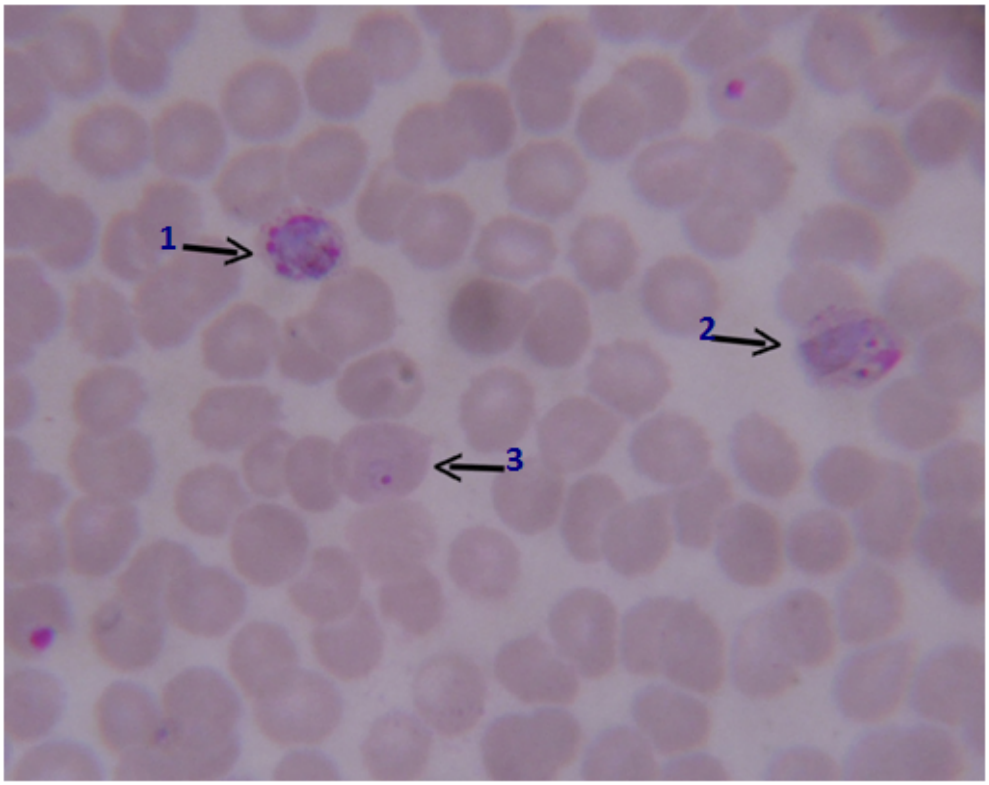


Figure (ix): Peripheral smear of the same patient showing Gametocyte (1) of Plasmodium falciparum.

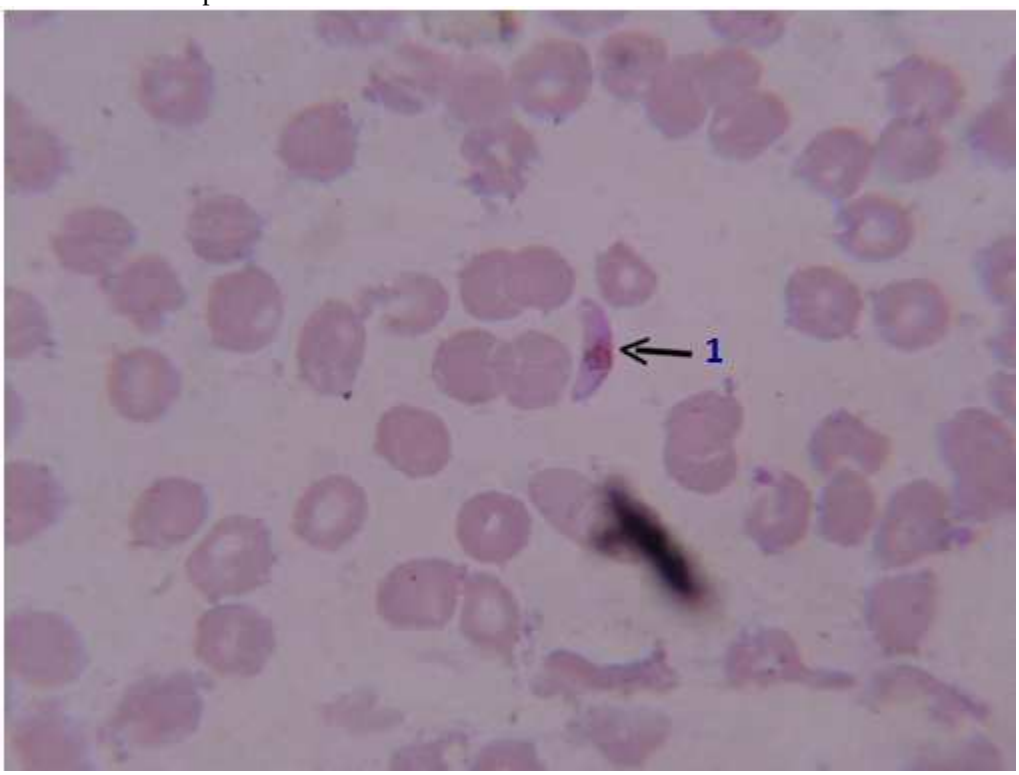


Figure (x): Peripheral smear of a 30 year male with fever for 10 days showing Gametocyte(1) of Plasmodium falciparum.

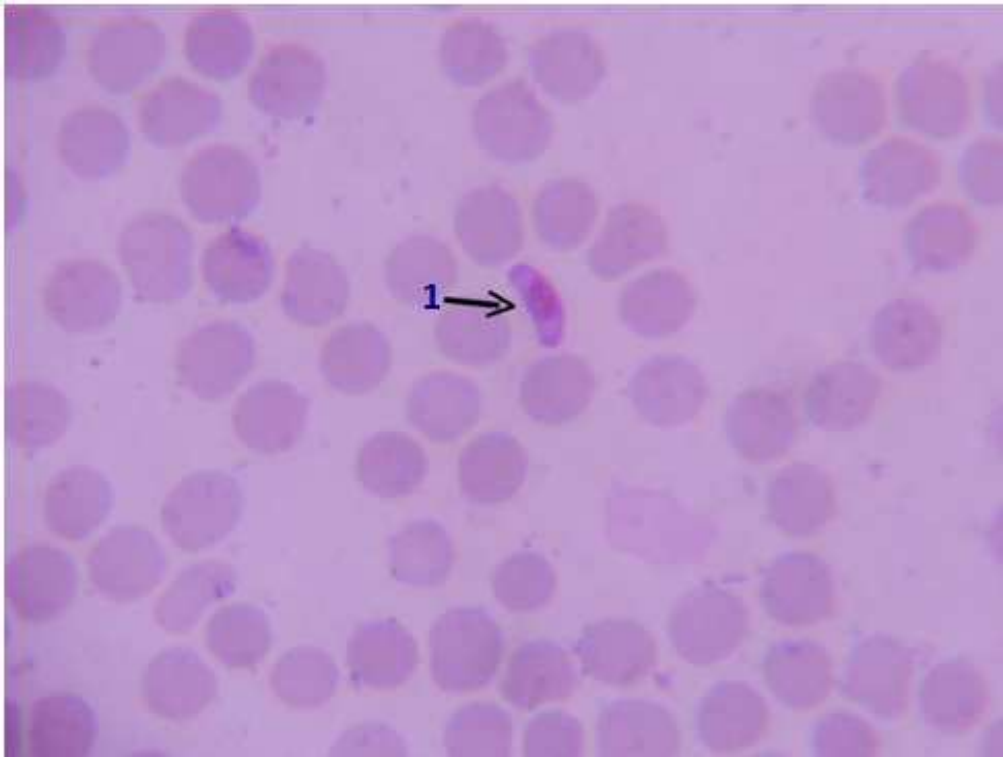


Figure (xi): Immunochromatography Kit

Figure (xii): ICT - Positive for non-falciparum malarial infection

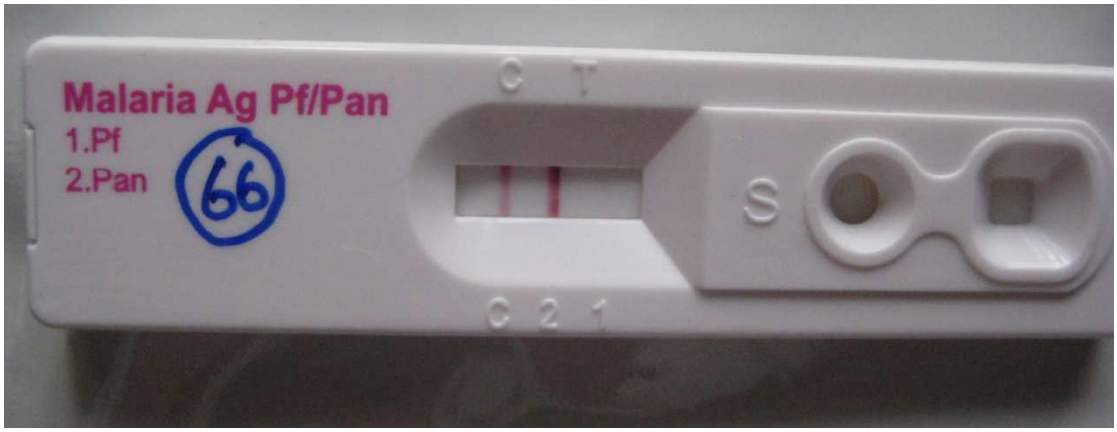


Figure (xiii): ICT - Positive for Plasmodium falciparum or mixed malarial infection

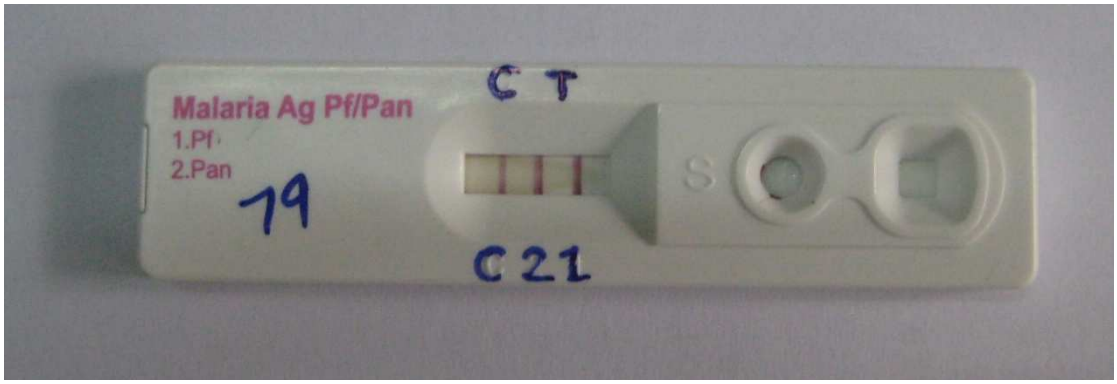


Figure (xiv): ICT - Positive for Plasmodium falciparum infection or persistance of pfHRP2

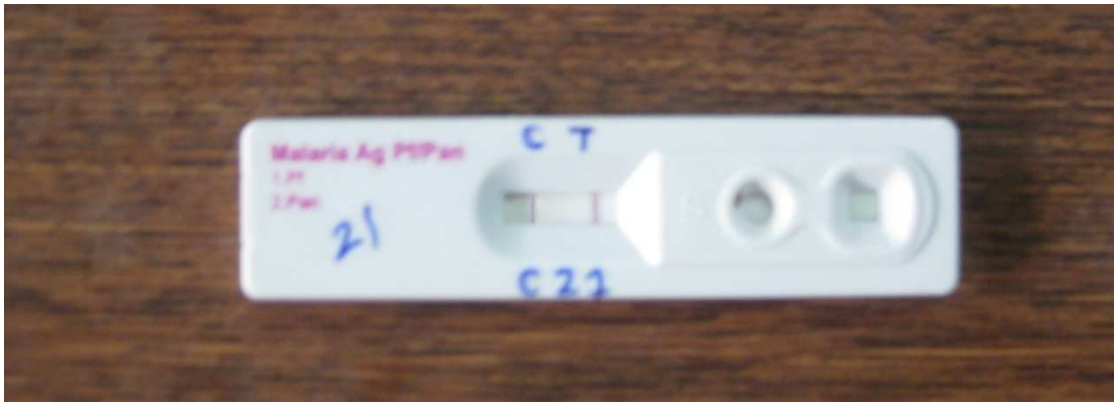


Figure (xv): ELISA kit for pan-malarial pLDH enzyme detection

Figure (xvi): Microtitre wells with controls and samples.
Sample 1,2 & 3 are positive for pLDH

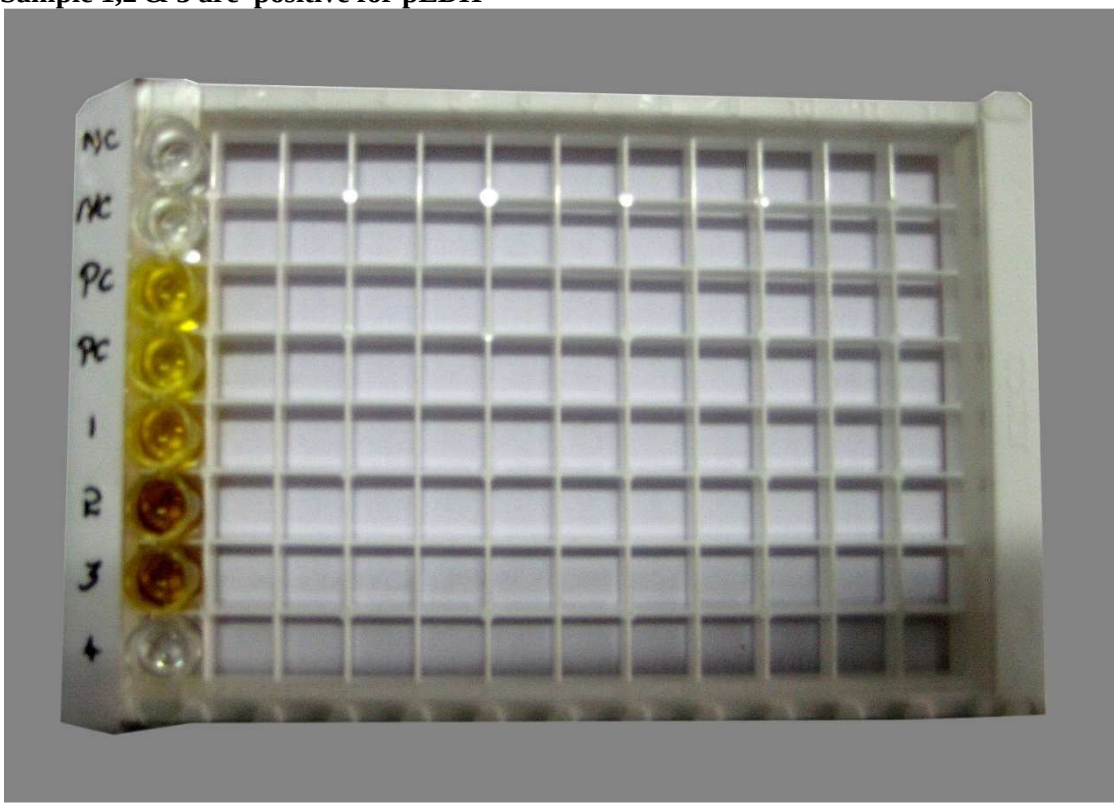


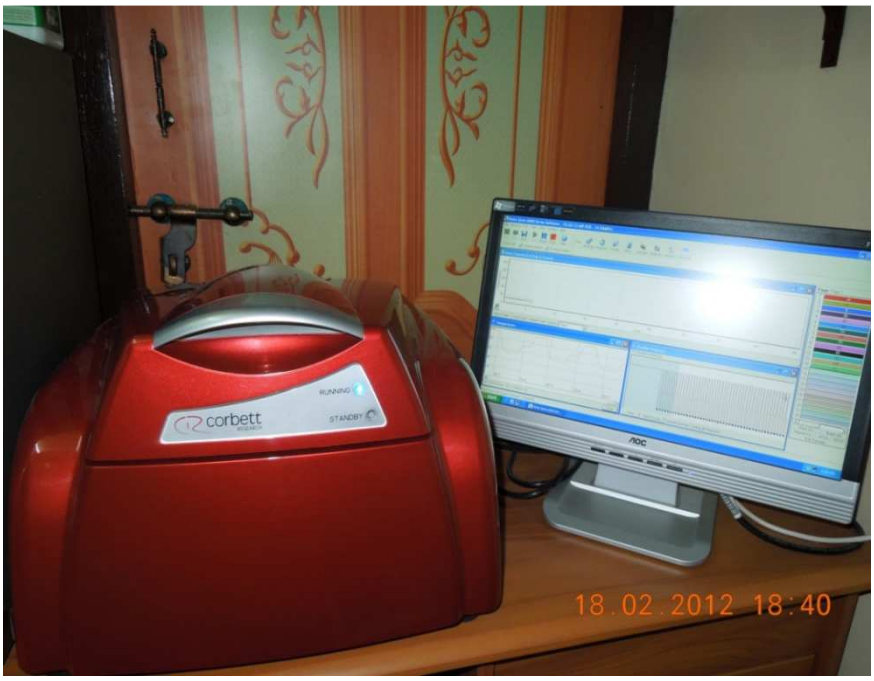
Figure (xvii): DNA extraction kit

Figure (Xviii): DNA extraction

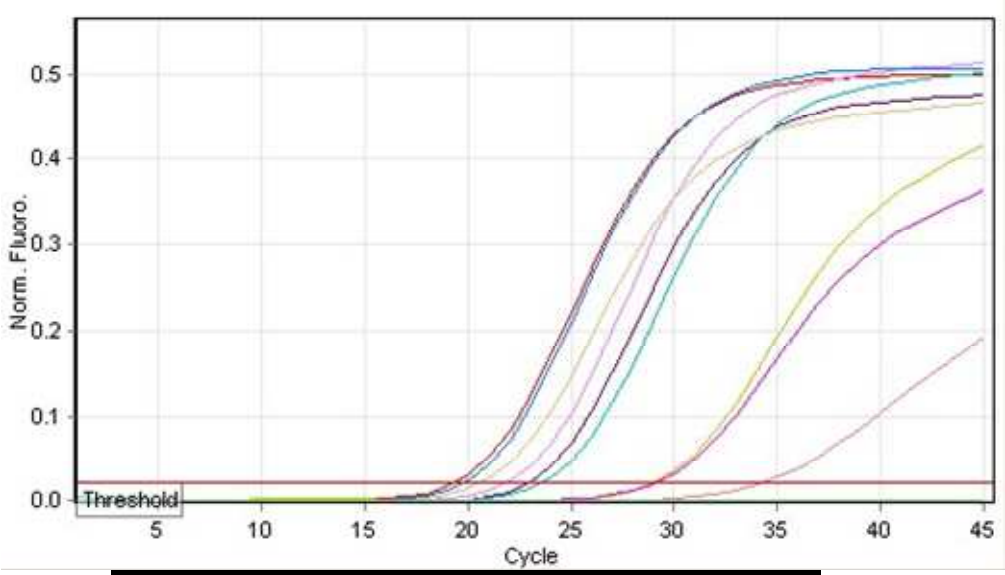
Figure (xix): PCR kit for detection of malaria.

Figure (xx): PCR instrument showing the rotor with master mix and DNA extract loaded vials

Figure (xxi): Samples loaded, programming done and the test run started



Figure(xxii): Report of Qualitative RT-PCR for malarial Parasite detection



No.	Colour	Name	Type	Ct
1		s1	Standard	19.46
2		1	Unknown	29.17
3		5	Unknown	NEG
4		36	Unknown	23.02
5		45	Unknown	21.97
6		66	Unknown	19.91
7		73	Unknown	NEG
8		74	Unknown	34.46
9		75	Unknown	NEG
10		80	Unknown	29.31
11		81	Unknown	NEG
12		86	Unknown	23.69
13		IAB	Unknown	20.76
14		nC	NTC	NEG

RESULTS

During the study period from September 2011 to September 2012, a total of 242 blood samples (EDTA added whole blood) were collected from the patients clinically suspected to have malaria. Peripheral smear was considered as the Gold standard test. Peripheral smear, ICT, ELISA were done on all samples. PCR was done with plasma on 40 selected samples (20 peripheral smear positive and 20 peripheral smear negative samples).

The statistical analysis of the qualitative data was done by using Statistical Package for Social Sciences (SPSS) version 17 software. P-values were obtained from Pearson Chi Square test.

ICT, ELISA and PCR were compared with the Gold Standard peripheral blood smear. Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were calculated and given with their estimate and 95% confidence interval.

The age of the patient under study ranged from 0 - 75 years. The commonest age group affected was 16 to 30 years (27.1%) **(Ref: Table 1)**.

The male: female ratio was 6:1 with 36 (85.71%) cases of male and 6(14.29%) cases of female **(Ref:Table 2)**. The highest incidence among

male was observed in the age group 16 to 30 years (18 cases), while among female, it was 0 to 15 years (4 cases) **(Ref: Chart 2)**.

Seasonal variation was observed in this study. Maximum number of cases were observed during the month of September 2011 - 6/23 cases (26%) and September 2012 - 8/33 cases (24%), followed by October 2011 - 7/33 (21%) cases and August 2012 - 5/25 (20%) cases **(Table 3)**.

Out of the 242 clinically suspected cases, 42 cases (17.4%) were positive for malarial parasite by thick and thin smear examination **(Table 4)**.

Of this, 39 were Plasmodium vivax, 2 were Plasmodium falciparum and one was a mixed infection with Plasmodium vivax and Plasmodium falciparum **(Table 4a)**.

Out of the 242 clinically suspected cases, 44 cases (18.8%) were positive for malarial parasite by Immunochromatography test **(Table 5)**.

Of this, 34 were found to be non-falciparum malarial infection, 8 were found to be either Plasmodium falciparum infection alone or a mixed infection of P.f with P.v / P.m / P.o and 2 were found to be either P.f infection or an indication of PfHRP2 persistence **(Table 5a)**.

When Microscopy and ICT were taken together to identify the species of the malarial parasite, 34 cases(14.05%) were identified as

Plasmodium vivax, 2 cases(0.83%) were identified as Plasmodium falciparum and 6 cases(2.48%) were identified as a mixed infection with Plasmodium vivax and Plasmodium falciparum **(Table 6)**.

Out of the 242 cases tested by ELISA method for malarial parasite specific LDH, 43 cases (17.8%) were found to be positive **(Table 7)**.

Out of 20 microscopy positive cases selected for PCR, all were positive for malarial parasite. Of the 20 microscopy negative cases selected for PCR 4 were positive **(Table 8)**.

When ICT was compared with the gold standard microscopy, the sensitivity was found to be 100%, specificity 99%, PPV 95.5% and NPV 100% **(Table 9)**.

When ICT was compared with the microscopy for the detection of P.f or mixed infection, ICT had a sensitivity of 100%, specificity 98%, PPV 37% and NPV 100% **(Table 10)**.

When ELISA (pLDH) was compared with the microscopy, ELISA showed a sensitivity of 100%, specificity of 99.5%, PPV of 97.7% and NPV of 100% **(Table 11)**.

When PCR was compared with microscopy for detection of malaria, the sensitivity, specificity, PPV and NPV were 100%, 80%, 83% and 100% respectively **(Table 12)**.

The confidence interval of sensitivity, specificity, PPV and NPV for ICT and ELISA were calculated and given in the **(Table 13)**. As PCR was not done on all the samples, confidence interval cannot be calculated for the sensitivity, specificity, PPV and NPV of PCR.

Table – 1
Age wise distribution of malaria positive cases

Age in years	**Total no. of cases**	**No. of cases Positive for malaria**	**Percentage (%)**
0 – 15	85	11	12.9%
16 – 30	**70**	**19**	**27.1%**
31 – 45	56	7	12.5%
46 – 60	27	5	18.5%
>60	4	0	0%
Total	242	42	17.3%

Table –2

Sexwise distribution of malaria positive cases(n=42)

Sex	No. of cases positive for malaria	Percentage (%)	Male:Female Ratio **6:1**
Male	**36**	**85.71%**	
Female	6	14.29%	

Table -3

Seasonal distribution of malaria from Sep'11-Sep'12

Month	Total no. of samples	No. of positives	Percentage (%)
2011			
September	**23**	**6**	**26%**
October	33	7	21%
November	30	4	13.3%
December	21	4	19%
2012			
January	9	0	0
February	8	0	0
March	8	0	0
April	5	0	0
May	13	2	16.6%
June	18	3	16.6%
July	16	3	18.7%
August	25	5	20%
September	**33**	**8**	**24%**

Table – 4

Results of microscopy

Total no. of Blood smears examined	Smears positive for malarial parasite			
	Thick smear	Thin smear	Total	%
242	42	42	42	17.4%

Table –4a

Distribution of Plasmodium species as identified by Thin smear (n=42)

Species	No. of positives	Percentage (%)
Plasmodium *vivax*	**39**	**92.8%**
Plasmodium f*alciparum*	2	4.8%
Mixed infection (P.v&P.f)	1	2.4%

Table –5

Results of Immunochromatography test

Total no. of samples	No. of positives	Percentage (%)
242	44	18.18%

Table –5a

Distribution of Plasmodium species as identified by ICT (n=44)

Species	No. of positives	Percentage (%)
Non-falciparum malarial infection	**34**	**77.3%**
Pf infection or mixed infection	8	18.2%
Pf infection or persistence of PfHRP2	2*	4.5%

Pf - Plasmodium *falciparum*

PfHRP2 – Plasmodium *falciparum* specific Histidine Rich Protein 2.

***** 2 cases indicating Pf infection or persistence of PfHRP2 was found to be due to persistence of PfHRP2.

Table -6

Distribution of Plasmodium species as identified by combination of Microscopy and ICT

Species	No. of positives by Microscopy	No. of positives by ICT	Microscopy & ICT taken together for speciation (n=42)	
			No.	%
Plasmodium vivax	39	34	**34**	**80.9%**
Plasmodium falciparum	2	8	2	4.8%
Mixed infection (P.v&P.f)	1		**6**	**14.3%**

Table –7

Results of ELISA(pLDH)

Total no. of samples tested	No. of positives	Percentage (%)
242	43	17.8%

Table –8

Positivity of malarial parasite by various technique among the 40 samples selected for PCR

Test	Positive by microscopy (n=20)	Negative by microscopy (n=20)
ICT positive	20	2
ELISA(pLDH) positive	20	**1**
PCR positive	20	**4**

Among the 20 microscopy negative samples selected for PCR, PCR has detected 4 cases as positive for malaria, ELISA has detected one case as positive for malaria which was also detected positive by PCR and 2 cases detected by ICT as PfHRP2 positive but pLDH negative, were negative by PCR.

Table -9
Cross tabulation of ICT results by Microscopy (n=242)

ICT	Microscopy		Total
	Positive	Negative	
Positive	42	2	44
Negative	0	198	198
Total	42	200	242

Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Pearson chi-square test		
				Value	df	p-value
100	99	95.5	100	228.690	1	0.000

df = degree of freedom.

ICT shows excellent sensitivity, specificity and Negative Predictive Value, with good Positive Predictive Value in detecting malarial infection when compared to microscopy. p-value is 0.000 (p-value should be less than 0.005). Thus it is statistically significant. This shows that ICT is more sensitive than microscopy.

Table – 10

Cross tabulation of ICT(Pf or mixed infection) by microscopy (n=242)

ICT(Pf or mixed infection)	Microscopy		Total
	Positive	Negative	
Positive	3	5	8
Negative	0	234	234
Total	3	239	242

Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Pearson chi-square test		
				Value	df	p-value
100	98	38	100	89.227	1	0.000

ICT shows excellent sensitivity and Negative Predictive Value, with good specificity in detecting P.f or mixed malarial infection when compared to microscopy. p-value is 0.000 (p-value should be less than 0.005). Thus ICT being more sensitive than microscopy in detecting mixed infection and Plasmodium *falciparum* is statistically significant.

Table-11

Cross tabulation of ELISA results by Microscopy (n=242)

ELISA	Microscopy		Total
	Positive	Negative	
Positive	42	1	43
Negative	0	199	199
Total	42	200	242

Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Pearson chi-square test		
				Value	df	p-value
100	99	98	100	235.190	1	0.000

ELISA shows excellent sensitivity, specificity and Negative Predictive Value, with good Positive Predictive Value in detecting malarial infection when compared to microscopy. p-value is 0.000 (p-value should be less than 0.005). Thus ELISA being more sensitive and specific than microscopy in detecting malarial infection is statistically significant.

Table -12

Cross tabulation of PCR results by microscopy
(n=40)

PCR	Microscopy		Total
	Positive	Negative	
Positive	20	4	24
Negative	0	16	16
Total	20	20	40

Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Pearson chi-square test		
				Value	df	p-value
100	80	83	100	26.667	1	0.000

PCR shows excellent sensitivity and Negative Predictive Value in detecting malarial infection when compared to microscopy. p-value is 0.000 (p-value should be less than 0.005). Thus PCR being more sensitive with excellent Negative Predictive Value is statistically significant.

Table –13

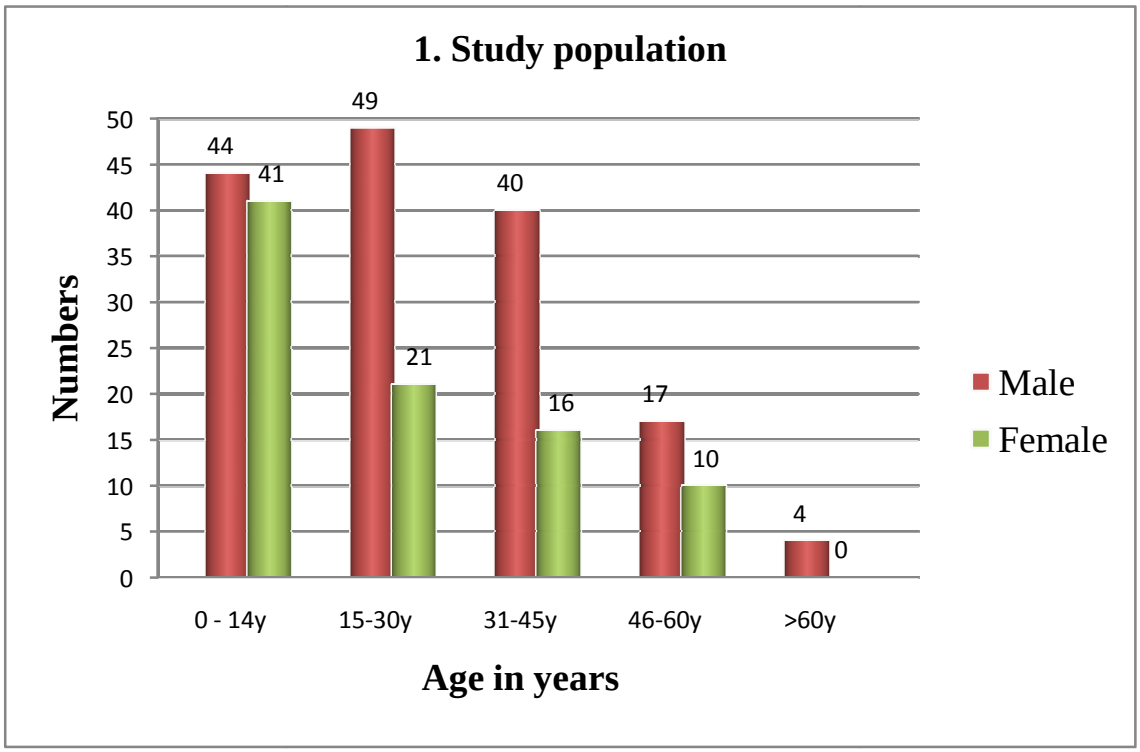
Evaluation of recent techniques in the diagnosis of malaria

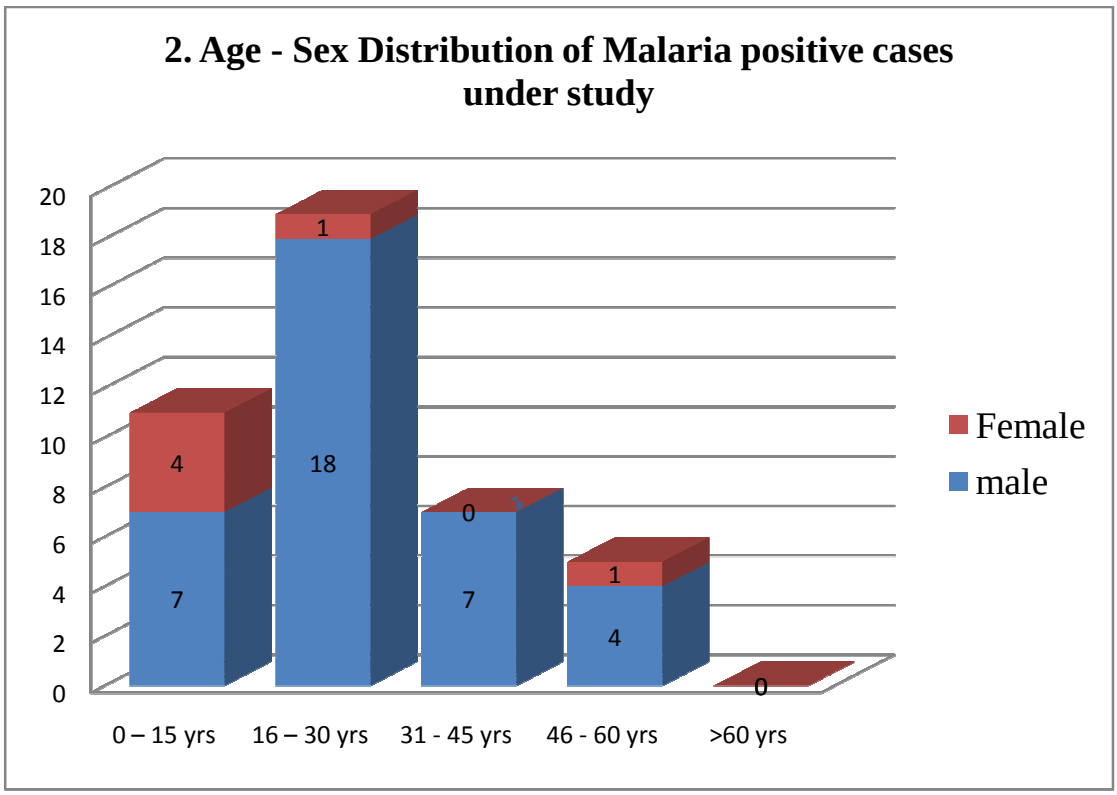
Test	Sensitivity(%) CI(%)	Specificity(%) CI(%)	PPV(%) CI(%)	NPV(%) CI(%)
ICT	100% (92 - 100%)	99% (96 – 100%)	95% (85 – 99%)	100% (98 – 100%)
ICT (Pf or mixed infection)	100% (44 – 100%)	98% (95 – 99%)	38% (14 – 69%)	100% (31 – 86%)
ELISA(pLDH)	100% (92 – 100%)	99% (97 – 100%)	98% (88 – 100%)	100% (98 – 100%)
PCR	100%	80%	83%	100%

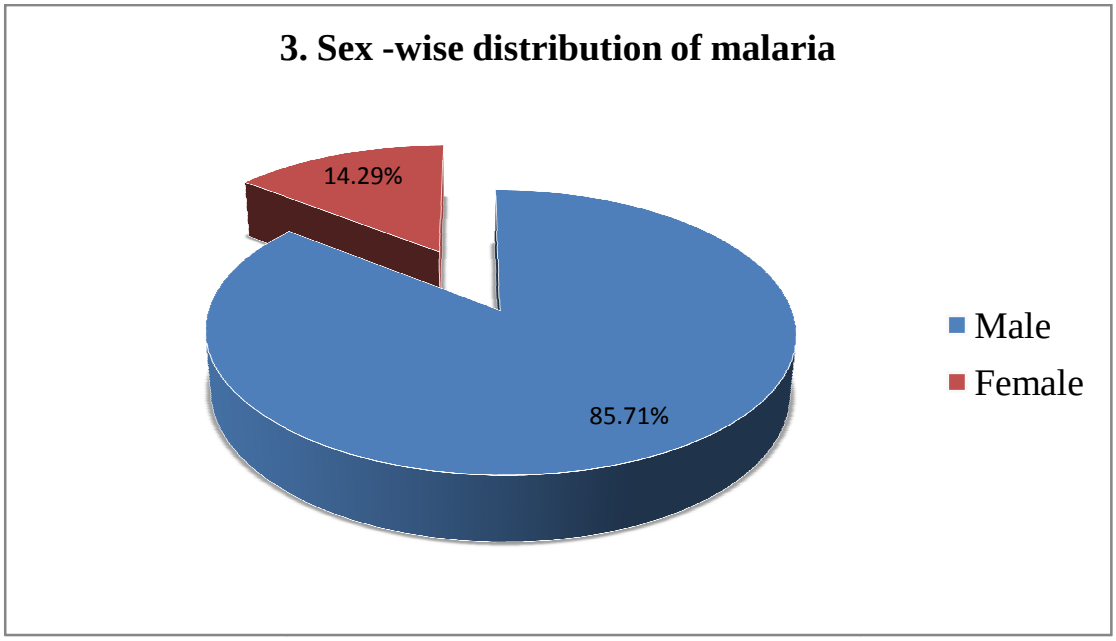
CI: 95% Confidence Interval.

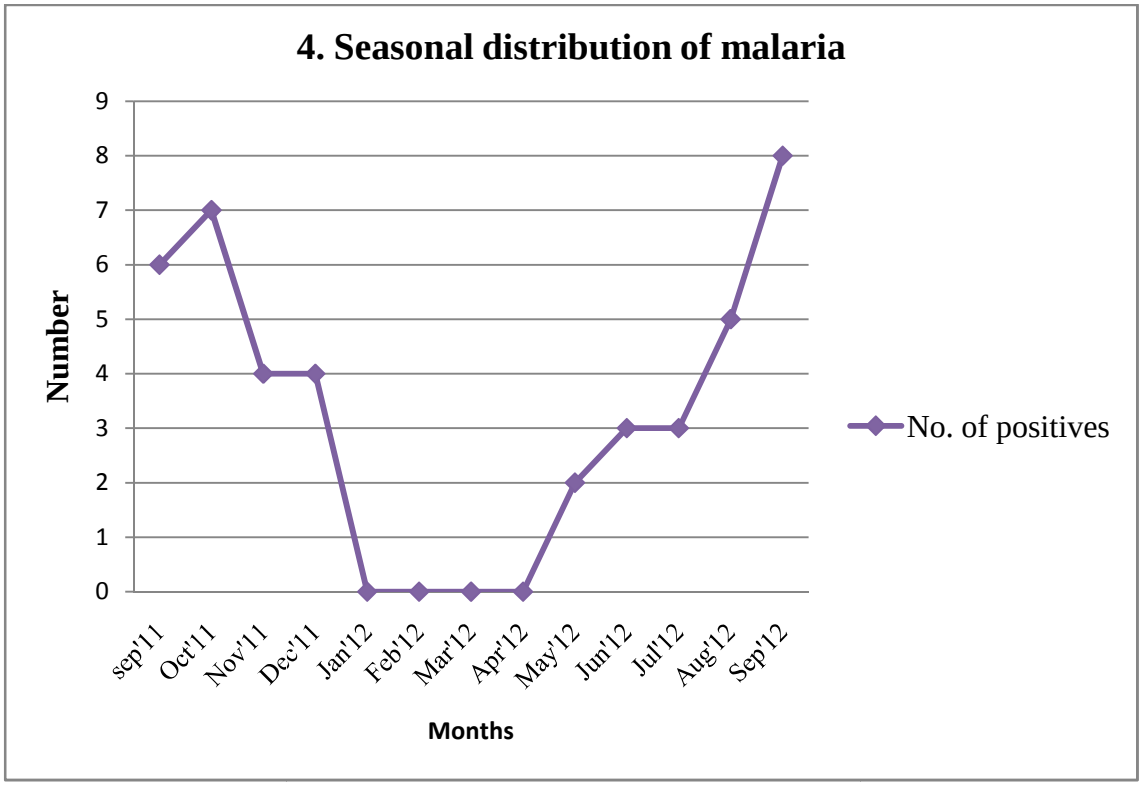
Thus, ICT and ELISA are equally sensitive (100%) and specific (99%) in detecting malarial infection. But ICT shows false positives, whereas ELISA do not. Of the three tests studied, PCR is the most sensitive (100%) with excellent Negative Predictive Value (100%).

PCR was not done on all the samples. Thus CI cannot be calculated.

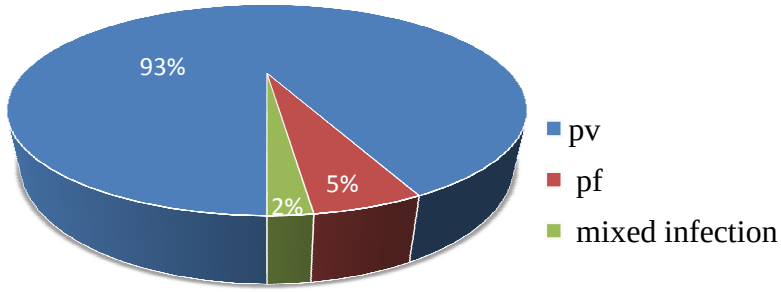

1. Study population
Numbers
50
45
40
35
30
25
20
15
10
5
0
44
41
49
21
40
16
17
10
4
0
0 - 14y
15-30y
31-45y
46-60y
>60y
Age in years
Male
Female


2. Age - Sex Distribution of Malaria positive cases under study
20
18
16
14
12
10
8
6
4
2
0
4
7
1
18
0
7
1
4
0
0 – 15 yrs
16 – 30 yrs
31 - 45 yrs
46 - 60 yrs
>60 yrs
Female
male

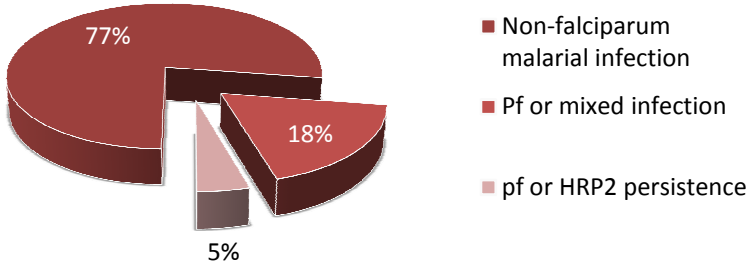

3. Sex -wise distribution of malaria
14.29%
85.71%
Male
Female


4. Seasonal distribution of malaria
Number
9
8
7
6
5
4
3
2
1
0
sep'11
Oct'11
Nov'11
Dec'11
Jan'12
Feb'12
Mar'12
Apr'12
May'12
Jun'12
Jul'12
Aug'12
Sep'12
Months
No. of positives

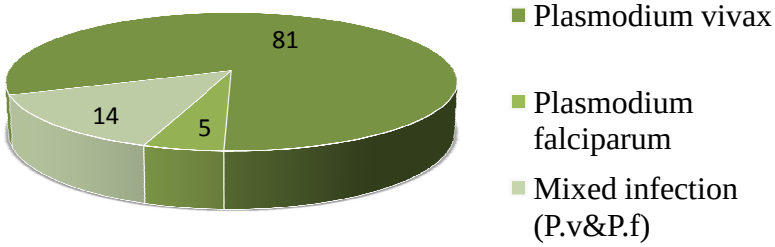
5. Distribution of species as identified by microscopy



6. Distribution of species as identified by ICT



7. Distribution of species as identified by taking microscopy and ICT together



DISCUSSION

In this study, blood samples from 242 clinically suspected cases of malaria were subjected to microscopy, ICT, ELISA. Out of 242 samples, PCR was performed on 40 samples.

Malarial parasite was detected in 42 cases out of the total 242 clinically suspected cases of malaria by both thick and thin smear (17.4%). Out of 42 cases, 39 were found to be Plasmodium *vivax* (92.8%), 2 were found to be Plasmodium *falciparum* (4.8%) and one was mixed infection with Plasmodium *vivax* and Plasmodium *falciparum* (0.41%).

The commonest age group affected was 16 to 30 years (27.1%). Males were found to be affected with malaria more than the females. P-value was 0.001($p<0.005$). Hence the difference in distribution of malaria positive cases in male and female was found to be statistically significant. The male: female ratio was 6:1. The reason for this was males were involved in more outdoor activities like agriculture and construction work. The females were mostly indoor and better clothed.

Seasonal variation was observed in this study. The peak incidence was observed in September and October which coincides with monsoon. Rainfall and increased humidity paves way for breeding and survival of mosquitoes.

Of the 42 positive cases, 12 were associated with history of travel to malaria endemic areas like Uttar Pradesh, Delhi, Bihar, Rajasthan, Orissa, Andhra Pradesh, Calcutta, and Chennai. This is because people from these places come to Coimbatore for business purpose or as contract workers for construction work. This also explains the common age group affected in this study and increase in incidence among males.

The incidence of malaria in this study is comparable with the following studies:

Parija *et al* at Pondicherry, India, in June 2009, reported 82 samples (19.95%) positive for malarial parasite out of 422 clinically suspected samples tested by peripheral smear [31].

The study by Bhat Sandhya k *et al* in 2012, reported 13.2% (66/500) of the cases positive by thick smear and 12% positive by thin smear examination in their study at Tumkur, Karnataka. Of this, 6.6% were Plasmodium *falciparum*, 4.6% were Plasmodium *vivax* and 0.8% was mixed infection [48].

Manjunath P Salmoni, *et al* at Bijapur , Karnataka, in 2011, had reported 15.5% (60/387) of cases positive by Leishman stained thick smear and 10.6%(41/381) of cases (P.v 48.8%, Pf 43.9%, mixed infection 7.3%) positive by Leishman stained thin smear [49].

A hospital based study by Kavitha Prabhu, *et al* at Davangere district of Karnataka, in 2010, reported 10.9% (22/202) cases positive for malaria (Pf 68.2%, Pv 13.6% and mixed Pv & Pf infection13.6%) [50].

Mahadev.S.Harani, *et al* , 2006, in their study at Karachi had reported 12.5% (72/560) of clinically suspected cases positive by Peripheral smear(Pf 32, Pv 29 and mixed infection with Pv & Pf 4 out of 560) [51].

In this study, the predominant species was identified as Plasmodium *vivax* which is comparable to the studies by Manjunath P Salmoni *et al* [49], A.H.Moon *et al* [52] and Shamim Akhtar *et al* [53].

Kavitha Prabhu *et al* [50], Mahadev.S.Harani *et al* [51] and Bhat Sandhya k, *et al* [48] in their studies had detected P. *falciparum* as the predominant species which is in contrast to this study.

Thus, the incidence and species distribution varies from country to country worldwide and also from region to region within a country.

ICT

In this study, out of 242 clinically suspected cases of malaria, 44 were positive for the presence of malarial parasite (18.18%) by ICT.

Of this 34 cases (77.3%) were non-falciparum malaria infection, 8 cases (18.2%) were found to be either Plasmodium *falciparum* alone or a mixed infection.

The disadvantage with the ICT used in this study is that, it cannot speciate the non-falciparum malarial infection and cannot distinguish a falciparum malarial infection from mixed infection of falciparum malaria with other Plasmodium species.

The 34 cases detected as non-falciparum malaria by ICT, were identified as asexual stages of Plasmodium *vivax* by peripheral smear. Thus the samples which were positive for pLDH (pan malarial antigen) i.e., samples which showed C & T2 lines by ICT were considered as Plasmodium *vivax*.

Eight ICT cases which showed positive for both pLDH and PfHRP2 were compared with microscopy for speciation. Out of 8 cases, two were identified as Plasmodium *falciparum* infection by microscopy. Thus these 2 cases were positive for P. *falciparum*. Six were positive for Plasmodium *vivax* by microscopy whereas the ICT showed Plasmodium *falciparum* in addition to Plasmodium *vivax*. Thus, these 6 cases were mixed infection of Plasmodium *vivax* and P.*falciparum*. Microscopy failed to detect Plasmodium *falciparum* infection in these 6 cases.

Two cases were positive for PfHRP2 alone by ICT. These cases, when looked into the history of the patient, were follow-up cases. Blood samples examined from these two cases, two weeks prior to this sampling

were positive for mixed infection and they showed clinical recovery with appropriate antimalarial treatment. In the follow-up sample (after 2 weeks of appropriate antimalarial treatment), pLDH had disappeared which indicated the response to treatment. PfHRP2 did not disappear because of its property of persisting in the blood for upto 28 days even after the disappearance of the parasite. Microscopy was negative for malarial parasite. Both the samples were negative for malaria by ELISA for pLDH and RT-PCR. Thus these 2 cases were due to persistence of the PfHRP2 enzyme and not Plasmodium *falciparum* infection.

Of the total malaria cases (42/242) detected in this study, 34 were Plasmodium *vivax*, 2 were Plasmodium *falciparum* and 6 were mixed infection with Plasmodium *vivax* and Plasmodium *falciparum* when microscopy and ICT were taken together for speciation.

The sensitivity of the ICT test to detect malarial parasite was 100%, specificity 99%, PPV 95.5% and NPV 100%. p - Value of ICT versus microscopy obtained by Pearson chi-square test was found to be 0.000 (p<o.oo5) and is found to be statistically significant.

There is a reasonable harmony between ICT and microscopy in detection of malarial parasite though there is variation in identifying the species. Similar results were obtained in the study Panchal H and

Desai Pratibha (2012) [54], Bhat Sandhya K *et al* (2012) [48] with SD Bioline malaria antigen kit.

This study revealed that ICT is highly sensitive in detecting the malarial parasite which is similar to the study by Wanna Chaijaroenkul *et al* (2010) [55].

ICT detected all Plasmodium *vivax* infection identified by microscopy. A study by Lt col MN Mishra and Surg capt RN Misra (2007) had reported a sensitivity and specificity of 100% for detection of Plasmodium *vivax* by ICT [29].

The following studies are contradictory to this study. Arsene Ratsimbasoa *et al* (2008) stated that SD bioline malaria Pf/pan test had much lower sensitivity (63%) in detecting non-falciparum malarial parasites [56]. A study by Heidi Hopkins *et al* (2007) [57] and Pankaj P.Taviad *et al* (2011) [58] had reported that pLDH was less sensitive (85%) but 100% specific in detecting the parasite. Mahadev S.Harani *et al* (2006), in their study had 89.7% sensitivity in detecting the Plasmodium *vivax* [51].

The diagnostic performance of ICT for Plasmodium *falciparum* or mixed infection was as follows: Sensitivity 100%, specificity 97.91%, PPV 37.5% and NPV 100%.

ICT is better than microscopy in identifying the mixed infection, especially the Plasmodium *falciparum* with other species. Out of 6 mixed infections, 5 were identified as Plasmodium *vivax* by microscopy and thus, it failed to detect Plasmodium *falciparum*. The reason for this is the character of the Plasmodium *falciparum* to sequester in the blood vessels of internal organs and lesser availability of the schizonts in the peripheral blood. Hence the parasite is not visible in the peripheral smear. In ICT, we are detecting the enzymes or proteins to identify the parasite - which is present in the blood even during the sequestration of the parasite. The following studies are comparable to this study.

In a Study by Wanna Chaijaroenkul *et al* (2011) [55], ICT Pf/Pan had detected more number of Plasmodium *falciparum* infection when compared to microscopy. A Study by Arsene Ratsimbasoa *et al* (2008) [56] reported sensitivity and specificity of 92.9% and 98.9% respectively for Plasmodium *falciparum* detection. Mahadev S.Harani *et al* (2006) [51], in their study had found a good diagnostic performance of 94.91% sensitivity, 96.21% specificity in detecting the Plasmodium *falciparum*. Praveen K Bharti *et al* in 2008 [59], in their study found that the overall sensitivity of RDT in detecting Plasmodium was 93% but, its accuracy in detecting non-falciparum malaria was less than that of detecting Plasmodium *falciparum*.

Kavitha Prabhu *et al* in 2010 [50], reported that ICT has the advantage of being highly sensitivity to detect the Plasmodium *falciparum* infection. In their study, out of 202 cases, 20 cases were positive for malarial parasite. ICT had detected 6 cases negative by microscopy. Of these 6 cases, 4 were identified as Plasmodium *falciparum* and 2 were false positives.

The complications of malaria were associated with mixed infection (Plasmodium *falciparum* and Plasmodium *vivax)* in this study. Out of the 6 patients diagnosed to have mixed infection 2 had cerebral malaria.

One of the patients with mixed infection was a10 year old male child. He presented with fever, haematuria and prostration. Blood investigation revealed mixed infection of Plasmodium *falciparum* with Plasmodium *vivax*, anaemia (Hb 6 g/dl) and thrombocytopenia (platelet count – 50,000/cumm). He was treated with antimalarial drugs (Artemesinin combination therapy), inspite of which fever persisted. Anaemia was corrected with blood transfusion. After 10 days, a follow up sample was tested for malaria. Peripheral smear was negative for malarial parasite. ICT showed disappearance of pLDH which means there was no live asexual stages of malaria parasite in the blood but, PfHRP2 persisted. ELISA for pLDH and RT- PCR were also negative for malaria. He was investigated for other causes of fever. Urine culture revealed growth of

Klebsiella *oxytoca* (on day 28 of illness). He was treated with appropriate antibiotics for one week but continued to have fever. A blood sample was again tested for malaria by microscopy and ICT methods (on day 38 of illness). Microscopy revealed ring stages and schizonts of Plasmodium vivax. ICT was positive for pLDH and there was no line in the PfHRP2 region. It is a relapse of Plasmodium *vivax* infection. Patient was treated with Chloroquine and Primaquine for which he responded and was discharged.

It is evident from this case that relapse occurs in Plasmodium *vivax* infection. Also, severe malaria predisposes to bacteremia especially Respiratory tract and urinary tract infections particularly in children as stated by Gordon C Cook and Alimuddin I Zumla [1].

Another patient among the six who were diagnosed to have mixed infection had anaemia (Hb 5g/dl) and splenomegaly who responded to antimalarials. Blood transfusion was given to correct anaemia.

Two patients with mixed infection did not have any complications and responded to antimalarials.

In mixed infection, the Plasmodium *falciparum* is the cause for complications because of its property of adherence to the blood vessels of internal organs like brain. ICT detected all the mixed infections (6/42). Peripheral smear detected only one mixed infection and detected other

five as Plasmodium *vivax* infection. ICT helped in diagnosing the mixed infections particularly with Plasmodium *falciparum* and thus the patient was treated appropriately.

ELISA

In this study, out of 242 clinically suspected cases of malaria, 43 were positive for malarial parasite (17.8%) by ELISA (pLDH).

The sensitivity, specificity, PPV and NPV of ELISA when compared with the gold standard was 100%, 99%, 97.7% and 100% respectively. Pearson chi-square test was done for ELISA vs. microscopy and p value was found to be 0.000 ($p<0.005$). Thus the better diagnostic performance of ELISA when compared to microscopy is found to be statistically significant.

One case which was detected positive for Plasmodium species by ELISA was negative by microscopy and ICT method. This patient presented to the medicine department with 10 days of fever. He was diagnosed outside to have malaria by ICT method (for malarial antibodies) and was treated with a course of chloroquine. The patient had persistant fever and was admitted in Coimbatore Medical College Hospital. He was investigated for the cause of fever. His peripheral smear was negative for malarial parasite. ICT was negative for pLDH and PfHRP2 antigens. ELISA for parasite specific pan malarial antigen was

positive. The sample was tested for ELISA (pLDH) in duplicates which were also positive. This was confirmed by RT-PCR. Speciation was not done by ELISA method in this study. The patient responded to antimalarial treatment. ELISA is more sensitive and specific especially in cases with persistance of infection due to low parasitemia.

RT-PCR

RT-PCR was done for 20 microscopy positive cases of malaria. These cases were also positive by ICT and ELISA. Seventeen cases which were negative for malaria by microscopy but highly suspected to have malaria clinically with other investigations for Pyrexia of unknown origin being insignificant were subjected to PCR. Two cases negative by microscopy and ELISA for malaria but, positive for PfHRP2 by ICT and One case positive for malaria by ELISA but negative by other two methods were also subjected to RT-PCR. All microscopy positive cases were positive for malaria by RT-PCR. The case which was positive for malaria by ELISA alone was positive by RT-PCR. Two cases positive for PfHRP2 by ICT was negative for malaria by RT-PCR. Of the 17 cases negative for malaria by microscopy, ICT and ELISA, 3 were detected positive by RT-PCR.

All the patients detected to have malaria by RT-PCR were treated with antimalarials. Patients responded to treatment.

The sensitivity of RT-PCR, when compared with the gold standard was 100%, specificity was 80%, PPV was 83.3% and NPV was 100%.

F.Perandin *et al* in 2004, had evaluated TaqMan-based real-time PCR for diagnosis of malaria. RT –PCR was compared with microscopy and nested PCR. The RT-PCR is highly sensitive (100%) and specific (100%) when compared to nested PCR for simultaneous detection of three Plasmodium species. Also it brought to light the errors in species identification by microscopic examination [60].

Bhavna Gupta in their study in 2010 at New Delhi, India, had reported a higher percentage of mixed infection was detected by PCR when compared to microscopy. In India, highest proportion of mixed infection was seen in Chennai samples [61].

Fracas *et al* in 2004 evaluated a real time PCR assay and reported a sensitivity of 99.5%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 99.6% for the detection of malaria when compared to nested PCR as the standard reference [41].

Brian J Taylor et al in 2011 conducted a study in Colombia. Here Real-time PCR was performed directly on whole blood and filter paper samples for detection of plasmodium DNA. The sensitivity and specificity ranged from 93 to 100% compared with the PCR performed on purified Plasmodium DNA [62].

Prapaporn Boonma et al, 2007, in their study at Thailand, compared three molecular methods with microscopy. Real-time PCR showed a sensitivity and specificity of 100 and 97% for P.*falciparum*, 87.5 and 93% for Plasmodium *vivax*, 50% and 99.2% for mixed infection. Real-time PCR was concluded as the best molecular method of the three (nested, multiplex and Real-time PCR) in their study [63].

Shokoples SE *et al in 2009*, in their study had stated that when RT-PCR was compared with microscopy, PCR demonstrated a greater sensitivity for the speciation of low level infections, mixed infections, and differentiation of "non-falciparum" species [64].

The above studies are comparable to this study in the sensitivity of detecting the malarial parasite. Speciation was not done by PCR in this study.

PCR, though has a high sensitivity in detecting malarial parasites, it has limitations in using as a routine diagnostic method in view of its cost, need for experienced person, continuous electricity supply and availability in all hospitals.

RT-PCR, when compared to the conventional PCR has a short turn-around time of only 3 hours and less chance of cross-over contamination. This shorter turnaround time could rapidly identify

undiagnosed or misdiagnosed cases, with an early, direct impact on patient management.

For a peripheral smear to be stained and examined thoroughly for malarial parasite, it needs a maximum of one hour. Speciation can be done and it is very cheap.

Though the peripheral smear has these advantages, it fails to detect Plasmodium *falciparum* infection when the parasites are sequestered in the blood vessels of internal organs and non-falciparum malarial parasites when the parasitemia is low.

In this study RT-PCR had detected 4 malaria positive cases which were not detected by the peripheral smear. The reason for failure to detect malarial parasite by peripheral smear was low parasitemia due to intake of antimalarial drug intake prior to submission of sample for investigation.

The cycle threshold (Ct) for the standard (concentration – 25000000 copies) used in this study was 19.46. The cycle threshold for the positive samples detected by microscopy was between 19.91 and 29.31. The cycle thresholds of the four samples negative by microscopy were 35.1, 37.0, 36.13 and 34.46.

Thus, RT-PCR because of its high sensitivity, it can be used as a second line diagnostic tool and as a confirmatory test in the diagnosis of malaria especially in a tertiary care hospital or reference laboratories with facilities to perform molecular methods as stated by S C Parija [65], Fracas *et al* [41], Berry A *et al* [66], Tham *et al* [67] and Stephanie P. Johnston *et al* [68].

SUMMARY

- This cross-sectional study was done over a period of 13 months and the study population comprised of 242 patients clinically suspected to have malaria.
- The commonest age group affected was 16 – 30 years.
- There was a male preponderance with male: female ratio of 6:1.
- Seasonal variation in the distribution of malaria was observed and maximum number of positive cases occurred in September and October.
- All the samples were subjected to microscopic examination of Leishman stained peripheral smear, Immunochromatography, ELISA for parasite specific pan-malarial LDH. RT-PCR was done on 40 samples.
- Microscopy detected 42 cases (17.4%), ICT was able to detect 44 cases (18.8%) and ELISA was able to detect 43 cases (17.8%).
- 20 cases detected positive by microscopy were also detected as positive for malarial parasite by RT-PCR. Of the 20 cases detected as negative by microscopy, RT-PCR detected 4 cases as positive for malaria.

- Microscopy detected 39 as Plasmodium *vivax*, two as Plasmodium *falciparum* and one as mixed infection with Plasmodium *vivax* and Plasmodium *falciparum*.
- ICT detected 34 cases as non-falciparum malaria and 8 cases as Plasmodium *falciparum* infection or mixed infection.
- When microscopy and ICT were taken together for speciation, 34 were identified as Plasmodium *vivax*, 2 as Plasmodium *falciparum* and 6 as mixed infection with Plasmodium *vivax* and Plasmodium *falciparum*.
- Plasmodium *vivax* was found to be the predominant species in Coimbatore.
- Complications due to malaria were commonly associated with mixed infection.
- Microscopy was taken as the gold standard test and ICT, ELISA and PCR were evaluated for their diagnostic performance.
- All the three recent diagnostic methods (ICT, ELISA and PCR) were found to be 100% sensitive when compared to microscopy in the diagnosis of malaria.
- Microscopy is cheaper and able to speciate but, it is labour intensive, needs expertise and time consuming when compared to

ICT. Also it failed to detect mixed malarial infection in 5 cases in this study.

- ICT is a rapid diagnostic test, simple to perform and does not need expertise. The diagnostic kit used in this study can be stored at room temperature away from direct sunlight. It is most suitable for field conditions.
- ICT detected the mixed infection of Plasmodium *vivax* and Plasmodium *falciparum* in 6 cases in this study. It can be used in all levels of health care centres as an adjunct to microscopy.
- Enzyme pLDH in ICT helps to detect response to treatment.
- The limitation of ICT is, it is costlier than microscopy and the persistence of PfHRP2 results in false positivity which occurred in 2 cases in this study.
- ELISA for pLDH has a good diagnostic performance in detection of malaria, when compared with microscopy. There was no false positivity. It detected a case of malaria which was negative both by microscopy and ICT. It can be used in blood banks where large numbers of samples have to be screened.
- RT – PCR is 100% sensitive in detection of malaria, when compared with microscopy. It is more sensitive than ICT and ELISA. It serves as a diagnostic tool in identifying cases with low

parasitemia and thus helps in appropriate management of the patient. The limitation is the cost, availability of equipment and need for specially trained technicians.

CONCLUSION

Though malaria is a disease of antiquity and there are many diagnostic methods from Peripheral smear to PCR, in many situations it is a diagnostic challenge to the physician because patients present with varied symptoms from simple fever to cerebral malaria. Also the typical fever pattern of malaria is masked due self-medication by the patients before consulting the physician. The availability of ‘over the counter drugs’ which includes antibiotics in our country makes the situation even more difficult. Antibiotics like sulpha drugs and fluoroquinolones have antimalarial effect and decrease the level of parasites in the peripheral blood thus, making it difficult to diagnose malaria by peripheral smear or ICT. ELISA and RT-PCR are better diagnostic methods in this situation. The advantages and limitations of different diagnostic methods of malaria have to be borne in mind and patients should be investigated thoroughly with appropriate diagnostic method, before claiming them negative for malaria.

From this study, it is evident that, the non-microscopic diagnostic methods have good diagnostic performance in detecting the malarial parasite. ELISA is more sensitive and specific with good Positive Predictive Value and excellent Negative Predictive Value. In view of its high sensitivity, it can be used in blood banks. The limitation with

ELISA method (detection of panLDH) used in this study is, it is not able to speciate. Speciation is necessary to decide the antimalarial drug to be prescribed to the patient. With ELISA for pLDH, it is possible to follow the patient's response to treatment. It also has the ability to detect malaria in cases of low parasitemia (below the level of detection by microscopy), especially when samples are submitted to the laboratory after initiation of antimalarial therapy.

RT-PCR shows an excellent sensitivity and Negative Predictive Value when compared to microscopy, thus allowing the exclusion of malaria in cases of Pyrexia of Unknown Origin in a tertiary care hospital. The turnaround time for RT-PCR is 3 hours which is rapid when compared to conventional PCR but when compared to microscopy and ICT, it is time consuming, needs equipments and round the time electricity supply in addition to an expertise to run the test. Limitations of RT-PCR performed in this study are inability to differentiate among four Plasmodium species and high cost per test when compared to microscopy and ICT.

ICT used in this study has advantages over ELISA and PCR in that it is able to detect Plasmodium *falciparum* and non-falciparum malarial infections. It is a rapid, highly sensitive and specific test with excellent Negative predictive value. It is superior to microscopy

in detecting Plasmodium *falciparum* or mixed infection of Plasmodium *falciparum* with other Plasmodium species. ICT can be used as an initial investigation in critically ill patients particularly when cerebral malaria is suspected in whom the parasites may be sequestrated. A limitation with this ICT is its inability to differentiate non-falciparum malarial infection and to differentiate whether it is Plasmodium *falciparum* infection alone or it is a mixed infection. ICT also shows false positives. Use of ICT and microscopy for all the samples in a health care centre would help in speciation and detecting all mixed infections. Thus ICT can be used as an adjunct to microscopy in the diagnosis of malaria in a tertiary care centre.

Thus non-microscopic method like ICT is a good adjunctive to microscopy in diagnosis of malaria, ICT and ELISA are good alternatives to microscopy in blood banks because of their high sensitivity and RT-PCR is the best method in diagnosis of malaria especially in cases of Pyrexia of Unknown Origin with parasitemia below the detection limit of peripheral smear.

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(i) LIST OF TABLES

(ii) LIST OF CHARTS

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1.	Study population
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3.	Sex -wise distribution of malaria
4.	Seasonal distribution of malaria
5.	Distribution of species as identified by microscopy
6.	Distribution of species as identified by ICT
7.	Distribution of species as identified by taking microscopy and ICT together

(iii) LIST OF COLOUR PLATES

S.NO.	COLOUR PLATES
i.	Peripheral smear - Thick and thin smear
ii.	Peripheral smear of a 1 year old male child with fever for a week showing early ring forms (1,2) of Plasmodium vivax.
iii.	Peripheral smear of a 45 year old male with fever for 10 days showing Ring forms (1,2,3) of Plasmodium vivax with Schuffner's dots and enlarged RBCs
iv.	Peripheral smear of a 51 year male with fever, rigors and headache for 5 days showing amoeboid form(2) and Early ring form (1) of Plasmodium vivax.
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vi.	Peripheral smear of a 54 year male with fever for 10 days showing early schizont(1) and early ring form (2) of Plasmodium vivax.
vii.	Peripheral smear of a 3 year old female child with cerebral malaria showing early schizonts(2), Trophozoite(1) and early ring form(3) of Plasmodium vivax with enlarged RBCs.
viii.	Peripheral smear of the same patient showing mature schizont (1), Trophozoite(2) and early ring form(3) of Plasmodium vivax and enlarge RBCs.
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xi.	Immunochromatography Kit
xii.	ICT - Positive for non-falciparum malarial infection

S.NO.	COLOUR PLATES
xiii.	ICT – Positive for Plasmodium falciparum or mixed malarial infection
xiv.	ICT – Positive for Plasmodium falciparum infection or persistence of PfHRP2
xv.	ELISA kit for pan-malarial pLDH enzyme detection
xvi.	Microtitre wells with controls and samples. Sample 1,2 & 3 are positive for pLDH
xvii.	DNA extraction kit
xviii.	DNA extraction
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xx.	PCR instrument showing the rotor with master mix and DNA extract loaded vials
xxi.	Samples loaded, programming done and the test run started
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(iv) LIST OF ABBREVIATIONS

WHO	World Health Organization
ELISA	Enzyme Linked Immunosorbent assay
RDT	Rapid Diagnostic Test
ICT	Immunochromatography
RT-PCR	Real Time - Polymerase Chain Reaction
LDH	Lactate Dehydrogenase
HRP	Histidine Rich Protein
NVBDCP	National Vector Borne Disease Control Programme
ATP	Adenosine Tri Phosphate
ACT	Artemesinin Combination Therapy
NIMR	National Institute of Malarial Research
QBC	Quantitative Buffy Coat
APDS	Automated parasite detection system
PMA	Pan Malarial Antigen
CSP	Circumsporozoite protein
TRAP	Thrombospondin Related Adhesive Protein
LSA	Liver Stage Antigen
MSP	Merozoite Surface Protein
P.v	Plasmodium *vivax*
P.f	Plasmodium *falciparum*
P.m	Plasmodium *malariae*
P.o	Plasmodium *ovale*
EDTA	Ethylene Diamine Tetra Aceticacid
IC	Internal Control
SPSS	Statistical Package for Social Sciences
PPV	Positive Predictive Value
NPV	Negative Predictive Value

(v) APPENDIX

Requirements for Proper Smear Preparation:

1) Clean grease free glass slide of size 75x25x1.5mm & a spreader
2) A blood drop of size 2 – 3 mm
3) The drop should be spread quickly and smoothly.
4) Smear should be dried.
5) Smear should be prepared within an hour of collection of blood.

Thin smear preparation:

1. Sample should be mixed well.
2. The side of the smear and the patient's ID number marked on the slide.
3. The slide should be kept on a flat surface, a 2-3 mm drop of mixed whole blood is placed on the slide about 1/4 inch from the right side of the slide, using the wooden applicator sticks held in the right hand.
4. The slide should be held securely in the left hand and the spreader slide held in the right hand between thumb and forefinger.
5. The spreader slide is placed onto the lower slide in front of the blood drop at an angle of 45^0C, and the slide pulled back until it touch the drop.

6. The blood is allowed to spread by capillary action almost to the edge of the lower slide.
7. The spreader slide is lowered to 30^0C and pushed forward to the left till the blood is exhausted using a rapid and even motion.
8. The film is then allowed to air dry.

Thick smear preparation:

1. Sample mixed well.
2. A big drop of blood is placed on a slide and spread with corner of another slide to form an area of a half-inch square and allowed to dry. The thickness of the film should be such that it allows newsprint to be read or the hands of the wrist watch to be seen through the dry preparation.

Constituents of Leishman's stain: 0.15 g of Leishman powder in 100ml of acetone free methyl alcohol.

ICT kit components:

1. Malaria Ag P.f/Pan test device
2. Assay diluent
3. Disposable sample applicator

ELISA kit components:

1. Microwells coated with monoclonal anti p-LDH antibody.
2. Positive control – Goat anti-mouse serum with stabilizer.

3. Negative control - Bovine serum albumin with stabilizer.
4. Enzyme conjugate – Streptavidin HRP conjugate.
5. Conjugate diluents – Buffered solution containing stabilizing proteins and preservatives.
6. Antibody reagent – Biotinylated anti-pLDH antibody.
7. Sample diluents – Buffered solution containing stabilizing proteins and 0.1% sodium azide as preservative.
8. Substrate – Contains Tetramethylbenzidine and hydrogen peroxide.
9. Wash buffer – contains surfactants.
10. Stop solution – Diluted sulphuric acid (0.3N).
11. Microwell holder.

Reagent preparation:

i. Wash buffer diluted 20 times with distilled water.

ii. Antibody reagent diluted 50 times with sample diluents.

iii. Enzyme conjugate diluted 50 times with conjugate diluent.

DNA Extraction Kit Components

Reagents

1. Proteinase K, lyophilized: One vial with 25 mg of lyophilized proteinase K is included. It has to be dissolved in 1.25 ml of nuclease-free water and Stored at -20^0C.
2. Tissue Lysis buffer

3. Binding buffer.
4. Washing buffer 1 (W1) is supplied in a concentrated form. Before the first use, 30 ml of absolute ethanol to be added and reconstituted.
5. Washing buffer 2 (W2) is supplied in a concentrated form. Before the first use, it should be reconstituted with 80 ml of absolute ethanol.
6. Elution buffer (EL): 10 mM Tris-Cl (pH 8.5).

Columns and tubes

Binding column tubes: 2 ml tubes (for filtration)

1.5ml tubes (for elution)

PCR kit components

Color Code	Contents
R1 Blue	MALARIA Super mix.
R2 Yellow	Mg Sol RT.
MALARIA-S1 Red	MALARIA Standard 1 1 X 10^5 copies/µl
MALARIA-S2 Red	MALARIA Standard 2 1 X 10^4 copies/µl
MALARIA-S3 Red	MALARIA Standard 3 1 X 10^3 copies/µl
MALARIA-S4 Red	MALARIA Standard 4 1 X 10^2 copies/µl
MALARIA-S5 Red	MALARIA Standard 5 1 X 10^1 copies/µl
W White	Molecular Grade Water
IC-1 (R3) Green	IC-1 RG (R3)

R = Reagents

S = Quantitation Standards

W = Molecular Grade Water

(vi) PROFOMA

ID No. : Date:

Patient details:

Name : Address:

IP/OP No. :

Age :

Sex : Occupation:

Current Symptoms:

Duration of present illness:

Fever :

Chills :

Sweating :

Headache :

Others :

H/O Blood transfusion :

H/O Travel to malaria endemic area :

Past H/O malaria :

Treatment (in the past and the type of Medicines taken):

Chloroquine :

Sulfadoxine/Pyrimethamine :

Primaquine :

Others(Quinolones) :

Investigations:

Peripheral smear (thick and thin smear)

ICT

ELISA

PCR

(vii) WORK SHEET

Sample - whole blood
(Collect venous blood in an EDTA containing vacutainer).

↓

Aliquot the blood sample for ELISA and PCR.

↓

Separate the plasma from EDTA added whole blood for PCR

↓

Prepare peripheral smear with whole blood, stain with Leishman's stain and examine the smear under microscope.

↓

Immunochromatography test (whole blood).

↓

Perform ELISA for pan malarial pLDH antigen detection
[within 24 hours of collection of blood (whole blood)].

↓

Perform RT-PCR for detection of Malarial DNA using plasma.
(On selected samples only)

MASTER CHART

ID.No.	Op/IP.No.	Name	Age/Sex	Fever with chills	H/O BT	Anti-malarials & quinolone treatment	H/O Travel	complic-ations	PS	ICT	ELISA	PCR
1	51915	Ramesh	34/M	+	-	-	+	-	Positive(schizonts) for P.v	C &T2	+	+
2		Suresh	54/M	+	-	-	-	-	Positive(ring stage & schizonts)for P.v	C &T2	+	+
3		Nithya	27/F	+	-	-	-	-	Negative	-	-	ND
4	50171	Pooja	2/FCh	+	-	-	-	-	Negative	-	-	ND
5	52604	Vasu	41/M	+	-	-	+	-	Negative	-	-	-
6	53599	Pradeep	10/MCh	+	-	-	-	-	Negative	-	-	-
7	53506	Bijay	19/M	+	-	-	+	-	Positive(schizonts) for P.v	C &T2	+	+
8	53237	Boomadevi	50/F	+	-	-	-	-	Negative	-	-	ND
9	53036	Murugan	20/M	+	-	-	-	-	Negative	-	-	ND
10	53732	Erman	35/M	+	-	-	+	+	Positive(schizonts) for P.v	C,T1&T2	+	+
11	50353	Srikmar	38/M	+	-	-	-	-	Negative	-	-	ND
12	53135	Murugan	25/M	+	-	-	-	-	Negative	-	-	ND
13	356782	Karthikeyan	25/M	+	-	-	-	-	Negative	-	-	ND
14	34522	Anandkumar	4/MCh	+	-	-	-	-	Negative	-	-	ND
15	54461	Chadru	10/MCh	+	-	-	+	+	Positive(schizonts) for P.v	C,T1&T2	+	+
16	54021	Chelladurai	40/M	+	-	-	-	-	Negative	-	-	ND

17	54298	Chinnathai	55/M	+	-	-	-	-	Negative	-	-	ND
18	54203	Ravikumar	30/M	+	-	-	-	-	Negative	-	-	ND
19	50225	Elisa	35/F	+	-	-	-	-	Negative	-	-	ND
20	54055	Selvam	45/M	+	-	-	-	-	Negative	-	-	ND
21	53732	Erman	35/M	+	-	-	-	-	Negative	C&T1	-	ND
22	54100	Arumugam	28/M	+	-	-	-	-	Negative	-	-	ND
23		Sundar	25/M	+	-	-	-	-	Positive(ring stage & schizonts) for P.v	C &T2	+	ND
24		Lipika	2 1/2/FCh	+	-	-	-	-	Negative	-	-	ND
25		Boomika	4/FCh	+	-	-	-	-	Negative	-	-	ND
26		Pappan	25/M	+	-	-	-	-	Positive(schizonts) for P.v	C &T2	+	+
27	56047	Dharshan priya	4/FCh	+	-	-	-	-	Negative	-	-	ND
28	56025	Nagalakshmi	37/F	+	-	-	-	-	Negative	-	-	ND
29		Angamuthu	2/MCh	+	-	-	-	-	Negative	-	-	ND
30	57933	Mariammal	6/FCh	+	-	-	-	-	Negative	-	-	ND
31	57439	Vijay	12/MCh	+	-	-	-	-	Negative	-	-	ND
32	57447	Poornachandrika	12/FCh	+	-	-	-	-	Negative	-	-	ND
33	58251	Megala	21/2/FCh	+	-	-	-	-	Negative	-	-	-
34	58317	Shravanthi	4/FCh	+	-	-	+	-	Positive(schizonts) for P.v	C,T1&T2	+	+
35	58156	Selvam	3/MCh	+	-	-	-	-	Negative	-	-	-

36	379761	Ajam	22/M	+	-	-	+	-	Positive(schizonts) for P.v	C &T2	+	+
37	54684	Chinnasamy	55/M	+	-	+	-	-	Negative	-	-	-
38		Kannan	35/M	+	-	-	-	-	Negative	-	-	ND
39		Ganesan	48/M	+	-	-	-	-	Positive (gametocyte stage) for P.f	C,T1&T2	+	+
40	54461	Chandru	10/MCh	+	-	-	+	+	Negative	C &T1	-	ND
41	58870	Valarmathy	20/F	+	-	-	-	-	Negative	-	-	ND
42	58380	Sathyam	55/M	+	-	-	-	-	Negative	-	-	ND
43	59392	Reshma	5/FCh	+	-	-	-	-	Negative	-	-	ND
44		Buvaneshpandi	18/F	+	-	-	-	-	Negative	-	-	ND
45		Lakhi	30/F	+	-	-	-	-	Positive(schizonts) for P.v	C &T2	+	+
46	58920	Rifana	12/FCh	+	-	-	-	-	Negative	-	-	ND
47	59507	Haridharni	14/FCh	+	-	-	-	-	Negative	-	-	ND
48	59607	Raju	37/M	+	-	-	-	-	Negative	-	-	ND
49	59400	Pandiyan	33/M	+	-	-	+	-	Positive(ring stage & schizonts) for P.v	C &T2	+	+
50	54321	Thangaraj	12/MCh	+	-	-	-	-	Negative	-	-	ND
51	59540	Suganya	12/FCh	+	-	-	-	-	Negative	-	-	ND
52	58810	Bavani	5/FCh	+	-	-	-	-	Negative	-	-	ND
53		David	53/M	+	-	-	-	-	Negative	-	-	ND
54	57931	Mariammal	3/FCh	+	-	-	-	-	Negative	-	-	ND

55		Harish	21/2MCh	+	-	-	-	-	Negative	-	-	ND
56	59550	Aruldevan	9/MCh	+	-	-	-	-	Negative	-	-	ND
57	59047	Siranjeevi	12/MCh	+	-	-	-	-	Negative	-	-	ND
58	60105	Akhim	40/M	+	-	-	-	-	Negative	-	-	ND
59		Shakthi	26/F	+	-	-	-	-	Negative	-	-	ND
60	60669	Mohammad Ali	39/M	+	-	-	-	-	Positive(ring stage & schizonts) for P.v	C &T2	+	+
61	61624	Akshai	13/MCh	+	-	-	-	-	Negative	-	-	ND
62	61872	Ajithkumar	36/M	+	-	-	-	-	Negative	-	-	ND
63	64499	Kalaiarasu	8/MCh	+	-	-	-	-	Negative	-	-	-
64		Shanthi	38/F	+	-	-	-	-	Negative	-	-	-
65		Ramrav	65/M	+	-	-	-	-	Negative	-	-	-
66	54461	Chandru	10/MCh	+	-	-	+	+	Positive(schizonts) for P.v	C &T2	+	+
67	61922	Rajamani	42/M	+	-	-	-	-	Negative	-	-	+
68	62002	Semaran	30/M	+	-	-	-	-	Negative	-	-	+
69	63052	Poojalakshmi	3/FCh	+	-	-	-	-	Negative	-	-	ND
70	62920	Poongodi	5/FCh	+	-	-	-	-	Negative	-	-	ND
71	63362	Gopalakrishnan	4/MCh	+	-	-	-	-	Negative	-	-	ND
72	61554	Karthikeyan	40/M	+	-	-	-	-	Negative	-	-	ND
73	64869	Nithish	4/MCh	+	-	-	-	-	Negative	-	-	-

74	64957	Padmavathy	26/F	+	-	-	-	-	Negative	-	-	+
75	65113	Ejas	5/MCh	+	-	-	-	-	Negative	-	-	-
76	644376	Meenakshi	35/F	+	-	-	-	-	Negative	-	-	-
77	64250	Rajamani	34/M	+	-	-	-	-	Negative	-	-	-
78	64145	Murugesan	35/M	+	-	-	-	-	Negative	-	-	-
79		Balaji	30/M	+	-	-	-	-	Positive (gametocyte stage) for P.f	C,T1&T2	+	+
80	66646	Thanubhadra	25/M	+	-	+	+	-	Negative	-	+	+
81	66566	Karthiga	1/FCh	+	-	-	-	-	Negative	-	-	-
82	66112	Sanjay	7/MCh	+	-	-	-	-	Negative	-	-	-
83	66433	Janaki Eswari	10/FCh	+	-	-	-	-	Negative	-	-	-
84	66111	Shakthivel	4/MCh	+	-	-	-	-	Negative	-	-	ND
85	65798	Jayalalitha	27/F	+	-	-	-	-	Negative	-	-	ND
86	66680	Karuppannan	18/M	+	-	-	-	-	Positive(schizonts) for P.v	C &T2	+	+
87	66780	Savithri	50/F	+	-	-	-	-	Negative	-	-	ND
88	66908	Anandkumar	30/M	+	-	-	-	-	Negative	-	-	ND
89	67007	Sathish Babu	18/M	+	-	-	-	-	Positive(schizonts) for P.v	C &T2	+	+
90	444501	Samira	8/FCh	+	-	-	-	-	Negative	-	-	ND
91	439288	Roshan	8/MCh	+	-	-	-	-	Negative	-	-	ND
92	437804	Sakila	10/FCh	+	-	-	-	-	Negative	-	-	ND

93	68733	Mubasina	12/FCh	+	-	-	-	-	Positive(schizonts) for P.v	C &T2	+	+
94	70972	Srilatha	30/F	+	-	-	-	-	Negative	-	-	ND
95	70948	Nandini	28/F	+	-	-	-	-	Negative	-	-	ND
96	71037	Mani	32/F	+	-	-	-	-	Negative	-	-	ND
97	70756	Linchy	30/F	+	-	-	-	-	Negative	-	-	ND
98	45542	Sathyamohan	40/M	+	-	-	-	-	Negative	-	-	ND
99	71518	Manikandan	10/MCh	+	-	-	-	-	Negative	-	-	ND
100	71129	Ramya	6/FCh	+	-	-	-	-	Negative	-	-	ND
101	71958	Arulmani	5/MCh	+	-	-	-	-	Negative	-	-	ND
102	71806	Hiran	25/M	+	-	-	-	+	Positive(schizonts) for P.v	C,T1&T2	+	+
103	68247	Ashwini	4/MCh	+	-	-	-	-	Negative	-	-	ND
104	480668	Karthikeyan	8/MCh	+	-	-	-	-	Negative	-	-	ND
105	481138	Monisha	9/FCh	+	-	-	-	-	Negative	-	-	ND
106	71737	Sandhiya	8/FCh	+	-	-	-	-	Positive(schizonts) for P.v	C &T2	+	+
107	72671	Sandhya	6/FCh	+	-	-	-	-	Negative	-	-	ND
108	71927	Keerthiga	5/FCh	+	-	-	-	-	Negative	-	-	ND
109	72354	Masilamani	26/F	+	-	-	-	-	Negative	-	-	ND
110	73073	Prakash	9/MCh	+	-	-	-	-	Negative	-	-	ND
111	73466	Nithya	5/FCh	+	-	-	-	-	Negative	-	-	ND

112	73848	Umera	7/FCh	+	-	-	-	-	Negative	-	-	ND
113	72815	Karyammal	60/F	+	-	-	-	-	Positive(schizonts) for P.v	C &T2	+	+
114	1016	Harjith	61/2/Fch	+	-	-	-	-	Negative	-	-	ND
115	652	Chandru	24/M	+	-	-	-	-	Negative	-	-	ND
116	911	Abhas Ali	30/M	+	-	-	-	-	Negative	-	-	ND
117	548	Ajith	3/MCh	+	-	-	-	-	Negative	-	-	ND
118	805	Vaishnavi	7/FCh	+	-	-	-	-	Negative	-	-	ND
119	4016	Kumutha	4/FCh	+	-	-	-	-	Negative	-	-	ND
120	4279	Ishwarya	21/2/FCh	+	-	-	-	-	Negative	-	-	ND
121	4212	Sweetha	3/FCh	+	-	-	-	-	Negative	-	-	ND
122	4142	Kumari	37/F	+	-	-	-	-	Negative	-	-	ND
123	7958	Anbarasu	4/MCh	+	-	-	-	-	Negative	-	-	ND
124	7973	Aravind	6/Mch	+	-	-	-	-	Negative	-	-	ND
125	8007	Preethi	21/F	+	-	-	-	-	Negative	-	-	ND
126	8092	Karthika	8/FCh	+	-	-	-	-	Negative	-	-	ND
127	7862	Kaligaraj	32/M	+	-	-	-	-	Negative	-	-	ND
128	8686	Siva	29/M	+	-	-	-	-	Negative	-	-	ND
129	8571	Rabiya	27/F	+	-	-	-	-	Negative	-	-	ND
130	8858	Sikkanthar	35/M	+	-	-	-	-	Negative	-	-	ND

131	14277	Mohammad	4/MCh	+	-	-	-	-	Negative	-	-	ND
132	14908	Lalith chandran	3/MCh	+	-	-	-	-	Negative	-	-	ND
133	15559	Sriram	6/Mch	+	-	-	-	-	Negative	-	-	ND
134	15893	Md Sameem	8/MCh	+	-	-	-	-	Negative	-	-	ND
135	13197	Buvaneshwari	28/F	+	-	-	-	-	Negative	-	-	ND
136	12836	Nagal	35/F	+	-	-	-	-	Negative	-	-	ND
137	12910	Jeganathan	25/M	+	-	-	-	-	Negative	-	-	ND
138	13112	Habibul	19/M	+	-	-	-	-	Negative	-	-	ND
139	19868	Veni	26/F	+	-	-	-	-	Negative	-	-	ND
140	19555	Priya	40/F	+	-	-	-	-	Negative	-	-	ND
141	19838	Boopathi	6/Mch	+	-	-	-	-	Negative	-	-	ND
142	20234	Subban	54/M	+	-	-	-	-	Negative	-	-	ND
143	20168	Mahali	44/M	+	-	-	-	-	Negative	-	-	ND
144	26535	Thulasiammal	40/F	+	-	-	-	-	Negative	-	-	ND
145	202075	Kanimozhi	38/F	+	-	-	-	-	Negative	-	-	ND
146	26031	Surya	25/F	+	-	-	-	-	Negative	-	-	ND
147	26862	Deva	35/M	+	-	-	-	-	Positive(ring stage with schuffner dots) for P.v	C &T2	+	ND
148	28818	Dhakshanamoorthy	50/M	+	-	-	-	-	Negative	-	-	ND
149	28869	Santhoshkumar	40/M	+	-	-	-	-	Negative	-	-	ND

150	28152	Bakyalakshmi	35/F	+	-	-	-	-	Negative	-	-	ND
151	30176	Ramkishore	32/M	+	-	-	-	-	Positive (ring stage, trophozoites with schuffner dots) for P.v	C &T2	+	ND
152	30076	Velu	40/M	+	-	-	-	-	Negative	-	-	ND
153	30280	Manoj	35/M	+	-	-	-	-	Negative	-	-	ND
154	30246	Sayed Ahmed	46/M	+	-	-	-	-	Negative	-	-	ND
155	30063	Thulasimani	23/F	+	-	-	-	-	Negative	-	-	ND
156	24640	Gokul	10/MCh	+	-	-	-	-	Negative	-	-	ND
157	31241	Seenu	16/M	+	-	-	-	-	Positive (ring stage, trophozoites with schuffner dots) for P.v	C &T2	+	ND
158	31253	Kittan	71/M	+	-	-	-	-	Negative	-	-	ND
159	31186	Arumugam	46/M	+	-	-	-	-	Negative	-	-	ND
160	31091	Dharshan	1/MCh	+	-	-	-	-	Negative	-	-	ND
161	31647	Venkatachalam	35/M	+	-	-	-	-	Negative	-	-	ND
162	31645	Varun	11/MCh	+	-	-	-	-	Positive(ring stage & schizonts) for P.v	C &T2	+	ND
163	31888	Papammal	52/F	+	-	-	-	-	Negative	-	-	ND
164	35963	Esthar	37/F	+	-	-	-	-	Negative	-	-	ND
165	35820	Rajammal	51/F	+	-	-	-	-	Negative	-	-	ND
166	34233	Amudha	1/FCh	+	-	-	-	-	Negative	-	-	ND
167	36568	Aarish	1/MCh	+	-	-	-	-	Positive(ring stage) for P.v	C &T2	+	ND

168	36870	Suhavil	6/MCh	+	-	-	-	-	Negative	-	-	ND
169	37926	Thulasimani	2/FCh	+	-	-	-	-	Negative	-	-	ND
170	37139	Laila	40/F	+	-	-	-	-	Negative	-	-	ND
171	37139	Shanthamani	33/F	+	-	-	-	-	Negative	-	-	ND
172	38124	Vivekanandan	49/M	+	-	-	-	-	Negative	-	-	ND
173	37712	Praveena	28/F	+	-	-	-	-	Negative	-	-	ND
174	38192	Muthusamy	53/M	+	-	-	-	-	Negative	-	-	ND
175	38323	Amitkumar	27/M	+	-	-	-	-	Positive(ring stage with schuffner dots) for P.v	C &T2	+	ND
176	43546	Divya	3/FCh	+	-	-	-	+	Positive for ring stage of P.v & gametocyte of P.f	C,T1&T2	+	ND
177	43632	Rangasamy	90/M	+	-	-	-	-	Negative	-	-	ND
178	41170	Saradamani	52/F	+	-	-	-	-	Negative	-	-	ND
179	43635	Poornima	6/FCh	+	-	-	-	-	Negative	-	-	ND
180	42926	Vadivel	35/M	+	-	-	-	-	Negative	-	-	ND
181	44082	Vijaya	50/F	+	-	-	-	-	Negative	-	-	ND
182	43677	Sundharam	21/M	+	-	-	-	-	Positive (ring stage, trophozoites with schuffner dots) for P.v	C &T2	+	ND
183	44047	Prabakaran	20/M	+	-	-	-	-	Negative	-	-	ND
184	44020	Karthi	11/MCh	+	-	-	-	-	Negative	-	-	ND
185	44349	Balamohan	35/M	+	-	-	-	-	Negative	-	-	ND

186	44021	Karthikeyan	9/MCh	+	-	-	-	-	Negative	-	-	ND
187	46412	Selvaraj	15/M	+	-	-	-	-	Negative	-	-	ND
188	338428	Nithish	11/2/MCh	+	-	-	-	-	Positive (ring stage, trophozoites with schuffner dots) for P.v	C &T2	+	ND
189	46381	Deepak kumar	30/M	+	-	-	-	-	Positive (ring stage, trophozoites with schuffner dots) for P.v	C &T2	+	ND
190	46384	Marappan	35/M	+	-	-	-	-	Negative	-	-	ND
191	46673	Karthikeyan	75/M	+	-	-	-	-	Negative	-	-	ND
192	46675	Rathamani	48/F	+	-	-	-	-	Negative	-	-	ND
193	46966	Chinnasamy	40/M	+	-	-	-	-	Negative	-	-	ND
194	47019	Srirashavan	1/MCh	+	-	-	-	-	Negative	-	-	ND
195	46966	Chinnasamy	40/M	+	-	-	-	-	Negative	-	-	ND
196	46851	Peter	35/M	+	-	-	-	-	Negative	-	-	ND
197	49213	Chinnasamy	30/M	+	-	-	-	-	Negative	-	-	ND
198	49330	Mariammal	45/M	+	-	-	-	-	Negative	-	-	ND
199	49624	Kamalesh	6/MCh	+	-	-	-	-	Negative	-	-	ND
200	49870	Vellayangiri	17/M	+	-	-	-	-	Negative	-	-	ND
201	50011	Boopathy	32/M	+	-	-	-	-	Positive(ring stage & schizonts) for P.v	C &T2	+	ND
202	50150	Sureshkumar	48/M	+	-	-	-	-	Positive(ring stage & schizonts) for P.v	C &T2	+	ND
203	50070	Hari	21/M	+	-	-	-	-	Negative	-	-	ND

204	49958	Safiya parveen	6/FCh	+	-	-	-	-	Negative	-	-	ND
205	50139	Bathruvin	29/M	+	-	-	-	-	Negative	-	-	ND
206	50178	Kasthuri	26/F	+	-	-	-	-	Negative	-	-	ND
207	49821	Rohit kumar	25/M	+	-	-	+	-	Positive(ring stage & schizonts) for P.v	C,T1&T2	+	ND
208	47334	Manjuladevi	23/F	+	-	-	-	-	Negative	-	-	ND
209	47415	Sathyapriyanka	19/F	+	-	-	-	-	Negative	-	-	ND
210	49099	Asrafi pradad	46/M	+	-	-	-	-	Negative	-	-	ND
211	50346	Radha	60/F	+	-	-	-	-	Negative	-	-	ND
212	51195	Babulu	23/M	+	-	-	+	-	Positive (ring stage, trophozoites with schuffner dots) for P.v	C &T2	+	ND
213	50780	Berendrakumar	26/M	+	-	-	-	-	Negative	-	-	ND
214	51444	Omm	6/MCh	+	-	-	-	-	Positive (trophozoites stage) for P.v	C &T2	+	ND
215	51521	Siyaram	22/M	+	-	-	-	-	Positive (ring stage, trophozoites with schuffner dots) for P.v	C &T2	+	ND
216	50278	Maheswari	27/F	+	-	-	-	-	Negative	-	-	ND
217	51684	Vishnupriya	30/F	+	-	-	-	-	Negative	-	-	ND
218	57878	Ganesh	11/2/MCh	+	-	-	-	-	Negative	-	-	ND
219	51606	Patchiyammal	55/F	+	-	-	-	-	Negative	-	-	ND
220	52500	Rajarajeswaran	23/M	+	-	-	-	-	Negative	-	-	ND
221	51731	Nagammal	45/F	+	-	-	-	-	Negative	-	-	ND

222	52069	Devadhe	51/M	+	-	-	+	-	Positive (ring stage, trophozoites with schuffner dots) for P.v	C &T2	+	ND
223	52194	Chellammal	57/F	+	-	-	-	-	Negative	-	-	ND
224	52116	Pramod Radhan	23/M	+	-	-	-	-	Negative	-	-	ND
225	52118	Marathal	25/F	+	-	-	-	-	Negative	-	-	ND
226	52337	Kumar	20/M	+	-	-	-	-	Negative	-	-	ND
227	52391	Ajaykumar	12/MCh	+	-	-	-	-	Positive (ring stage, trophozoites with schuffner dots) for P.v	C &T2	+	ND
228	53225	Iyyappan	37/M	+	-	-	-	-	Negative	-	-	ND
229	53073	Balraj	33/M	+	-	-	-	-	Negative	-	-	ND
230	53287	Subramani	37/M	+	-	-	-	-	Negative	-	-	ND
231	53448	Chinnasamy	53/M	+	-	-	-	-	Negative	-	-	ND
232	53523	Gowri	22/F	+	-	-	-	-	Negative	-	-	ND
233	53608	Babukumar	24/M	+	-	-	-	-	Positive (ring stage, trophozoites with schuffner dots) for P.v	C &T2	+	ND
234	53608	Ambi	37/M	+	-	-	-	-	Negative	-	-	ND
235	53544	Mahalakshmi	31/F	+	-	-	-	-	Negative	-	-	ND
236	53647	Maran	30/M	+	-	-	-	-	Positive (ring stage, trophozoites with schuffner dots) for P.v	C &T2	+	ND
237	53547	Manikandan	24/M	+	-	-	-	-	Negative	-	-	ND
238	53668	Krishnamoorthy	35/M	+	-	-	-	-	Negative	-	-	ND

239	53893	Sasikumar	28/M	+	-	-	-	-	Negative	-	-	ND
240	53850	Ravindrakumar	21/M	+	-	-	-	-	Positive (ring stage, trophozoites with schuffner dots) for P.v	C &T2	+	ND
241	53900	Ashwin	11/2/MCh	+	-	-	-	-	Negative	-	-	ND
242	53930	Nihal	3/FCh	+	-	-	-	-	Negative	-	-	ND

(ix) KEY TO MASTER CHART

Sex:

M – Male

F – Female

BT – Blood Transfusion

PS – Peripheral smear

P.v – Plasmodium *vivax*

P.f – Plasmodium *falciparum*

ICT – Immunochromatography test

C- Control line

T1 – Test line 1

T2 – Test line 2

ELISA – Enzyme Linked Immunosorbent Assay

PCR – Polymerase Chain Reaction

ND – Test not done.

(x) CONSENT FORM

Dr G Lokeshwari, Post Graduate student in the Department of Microbiology, Coimbatore Medical College, Coimbatore, is studying on 'A Review of Alternatives to Conventional Microscopy in the Laboratory Diagnosis of Malaria'- regarding.

I am, _____ of age _____ years, sex - M/F, have been explained about the study. The venepuncture procedure, the risk involved and complications of the procedure were explained to me in my mother tongue. I have also been explained that the data obtained herein may be used for research and publication. I whole heartedly give my consent to participate in the study.

Place:

Date: Signature

A Review of Alternatives to Conventional Microscopy in the Laboratory Diagnosis of Malaria

ABSTRACT:

Background: Malaria is the disease of antiquity, having been recognized since Vedic times in India [1,2]. It is distributed worldwide with a great socioeconomic impact on the countries with intense transmission particularly in tropical regions [3]. Patients affected with malaria present with varied clinical picture from fever and malaise to life threatening symptoms like cerebral malaria. Diagnosing malaria based on clinical features alone may lead to over-diagnosis especially in endemic areas. Thus parasite based diagnosis is recommended for all patients by WHO. This study is focused on alternatives to conventional microscopy in the laboratory diagnosis of malaria.

Objectives: 1.To identify malarial parasite in patients with low parasite densities. 2. To compare the sensitivity and specificity of Rapid Diagnostic Test (RDT) with conventional microscopy. 3. To evaluate the diagnostic performance of pLDH Antigen detection ELISA. 4. To evaluate the role of PCR in the diagnosis of malaria.

Materials and Methods: This is a cross-sectional study conducted over a period of one year from September 2011 to September 2012. The study group included patients of all age groups and both sexes who were clinically suspected to have malarial fever (both inpatients and outpatients). Blood samples from these 242 patients were collected in a vacutainer with EDTA anticoagulant. Leishman's staining of the Peripheral smear, Rapid diagnostic test and ELISA for parasite specific pan malarial antigen (LDH) detection were done on all the samples. PCR was done with plasma for a total of 40 samples (20 microscopy positive and 20 microscopy negative samples).

Results: Peripheral smear was considered as the Gold standard test. ICT, ELISA and PCR were compared with the Gold Standard peripheral blood smear. Microscopy detected 42 cases (17.4%), ICT was able to detect 44 cases (18.8%) and ELISA detected 43 cases (17.8%). ICT detected mixed infection of Plasmodium falciparum with other Plasmodium species in 6 cases whereas microscopy detected only one mixed infection. RT-PCR detected 4 cases as positive for malaria among the 20 microscopy negative samples. All the three recent diagnostic methods (ICT, ELISA and PCR) were found to be 100% sensitive when compared to microscopy in the diagnosis of malaria.

Conclusion: Of the diagnostic methods studied, non-microscopic method like ICT is a good adjunct to microscopy in diagnosis of malaria, ICT and ELISA are good alternatives to microscopy in blood banks and RT-PCR is the best method in diagnosis of malaria especially in cases of Pyrexia of Unknown Origin with low parasitemia i.e., below the detection limit of peripheral smear.

Keywords: Malaria, ICT, ELISA, RT-PCR.